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**13. ABSTRACT** ***(Maximum 200 Words)***

Tumor invasion is a major obstacle to effective clinical management of breast cancer. To identify new targets for anti-invasive therapies, we have focused on the mechanisms by which the cell adhesion molecule E-cadherin suppresses tumor invasion. A related cadherin, N-cadherin, does *not* suppress cell movement, even though it is as effective as E-cadherin at mediating adhesion. We analyzed deletion mutants and exploited the difference between E- and N-cadherin to define regions of E-cadherin required for suppression of movement. We localized the key region that differs between E-cadherin and N-cadherin to a region consisting of the transmembrane segment and a small portion of the cytoplasmic domain, but demonstrated that E-cadherin does not regulate motility through sequestering p120, at physiological levels of expression. We also found that the catenin-binding domain is also required. Further, we identified two components that are tyrosine phosphorylated after E-cadherin contact, but determined they play no role in suppression of motility. We developed a new assay for analyzing the effect of cadherins on cell movement, which revealed that E-cadherin, but not N-cadherin, suppresses movement in intact monolayers of cells.

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## INTRODUCTION

Failures in treatment of breast tumors generally result from complications caused by tumor invasion and metastasis. This project aimed to analyze mechanisms that regulate movement and invasion of mammary epithelial cells, with the ultimate goal of developing new anti-invasive therapies. One causal event in the acquisition of invasive capacity during breast tumor progression is loss of the cell-cell adhesion molecule, E-cadherin. We found previously that the ability of E-cadherin to suppress cell movement and invasion is not directly related to its adhesive activity. Instead, we hypothesized that cell-cell contact mediated by E-cadherin generates signals that suppress cell movement. During this project period, we aimed to test this hypothesis and identify components of the E-cadherin signaling system.

## BODY

In Year 4 we made substantial progress toward our overall goals, in three major areas:

- We used digital time-lapse recording and quantitative image analysis methods to develop precise descriptions of the effects of E- and N-cadherin on the movement of breast cancer cells.
- We analyzed several deletion mutants of E-cadherin. The results confirmed that the juxtamembrane region is not required, but that the catenin-binding domain is required for cell contact suppression of movement. In collaboration with Ted Meigs and Patrick Casey, we found that binding of activated heterotrimeric G proteins of the G12 subfamily reverses E-cadherin adhesion and suppression of motility (Meigs et al., 2002), but deletion of the Gα12 binding site abolishes suppression without affecting adhesion (unpublished observation).
- Several laboratories have recently suggested that cell contact controls movement through regulation of the cellular localization of p120 (Anastasiadis et al., 2000; Anastasiadis and Reynolds, 2000; Grosheva et al., 2001; Noren et al., 2000). Our ongoing analysis of E-cadherin deletion mutants suggested, however, that this was unlikely to be the case. We therefore directly tested this suggestion, demonstrating that motility is not regulated by cellular localization of p120, at physiological levels of expression.

Our overall objectives and accomplishments are summarized below.

## REVISED TECHNICAL OBJECTIVES

**Technical Objective 1:** To verify that E- and N-cadherin differ in their ability to suppress invasion of mammary carcinoma cells and to use this difference to define regions of E–cadherin that are essential for suppressing invasion.

**Task 1.** Months 1-3. We will assay the invasiveness of MDA-MB-435 cells and verify their N-cadherin expression.

**Task 2.** Months 3-9. MDA-MB-435 cells and MDA-MB-231 cells will be transfected with control, E-cadherin, N-cadherin, chimeric E/N cadherin vectors and with E-cadherin mutant vectors. Permanent lines will be selected, re-cloned, and characterized for cadherin expression and adhesion.

**Task 3.** Months 9-15. The invasion and motility of the transfected lines will be evaluated. Each assay will be performed 3-5 times.

**Technical Objective 2** To determine whether an intact juxtamembrane domain is required for E-cadherin-induced tyrosine phosphorylation.

**Task 1.** Months 4-10. Work out assays and then complete final analyses of tyrosine phosphorylation in untransfected MDA-MB-435 cells and MDA-MB-231 cells.
**Task 2.** Months 12-24. Assay tyrosine phosphorylation in MDA-MB-435S cells and MDA-MB-231 cells transfected with full-length or mutant E-cadherins.

**Technical Objective 3** To identify factors that interact with the juxtamembrane domain of E–cadherin.

***(Task 1 and Task 2.** These experiments were duplicative of the NIH award and were deleted).*
**Task 3.** Months 4-10. We will analyze, by co-immunoprecipitation studies, components that may be associated with E-cadherin, but not the JM-deleted form, in MDA-MB-435 cells and MDA-MB-231 cells.
**Task 4.** Months 10-24. If associated proteins are seen, attempts will be made to identify them by using antiserum to candidate proteins or by excision from gels and microsequencing.
**Task 5.** Months 6-15. We will screen yeast two-hybrid libraries for additional positive colonies. The clones will be purified and identified by sequencing.
**Task 6.** Months 16-36. We will test the physiological relevance of interactions between components encoded by the new clones and we will define the sequences needed for interaction.
**Task 7.** Months 9-18. We will use GST-fusion proteins for affinity-purification of components associated with the JM domain.
**Task 10.** Months 18-24. We will produce antibodies against the affinity-purified components and begin testing for physiological interactions.
**Task 11.** Months 18-36. We will attempt to identify proteins that are tyrosine phosphorylated in response to E-cadherin binding and will begin to test whether they interact with E-cadherin.

## SUMMARY OF WORK ACCOMPLISHED ON REVISED TECHNICAL OBJECTIVES

**Technical Objective 1:** To verify that E- and N-cadherin differ in their ability to suppress invasion of mammary carcinoma cells and to use this difference to define regions of E–cadherin that are essential for suppressing invasion.

***(Previously reported)** For most of our experiments, we have used an isolate of the human breast cancer cell line MDA-MB-435 that is motile and does not express N-cadherin, E-cadherin, or P-cadherin. We verified the motility of this isolate and produced permanently transfected lines of MDA-MB-435 that express full-length E- or N-cadherin and seven E/N chimeric constructs. Some constructs were also expressed in the MDA-MB-231 breast cancer cell line. We completed the characterization of cadherin expression and adhesion of these lines Objective 1, task 1).*

*We found that E- and N-cadherin are similar in adhesive activity in in vitro assays, yet E-cadherin suppresses movement of MDA-MB-435 breast cancer cells, while N-cadherin does not. Thus, adhesion alone is not sufficient to suppress motility, implying that E-cadherin performs some additional function that N-cadherin does not. To define the region of E-cadherin required to suppress motility, we analyzed the E/N chimeric cadherins (Objective 1, tasks 2 and 3). For each of the chimeric cadherin constructs, multiple, independent clones with similar levels of cadherin expression were examined in the wound-filling assay. Consistent results were obtained and are summarized in the table on the following page.*

| Cadherins | Transmembrane segment | Cytoplasmic segment | Suppression of motility |
|---|---|---|---|
| E-cadherin, NEE | E | E | Yes |
| EEN, NEN | E | N | Yes |
| NNE, ENE | N | E | Yes |
| N-cadherin, ENN | N | N | No |

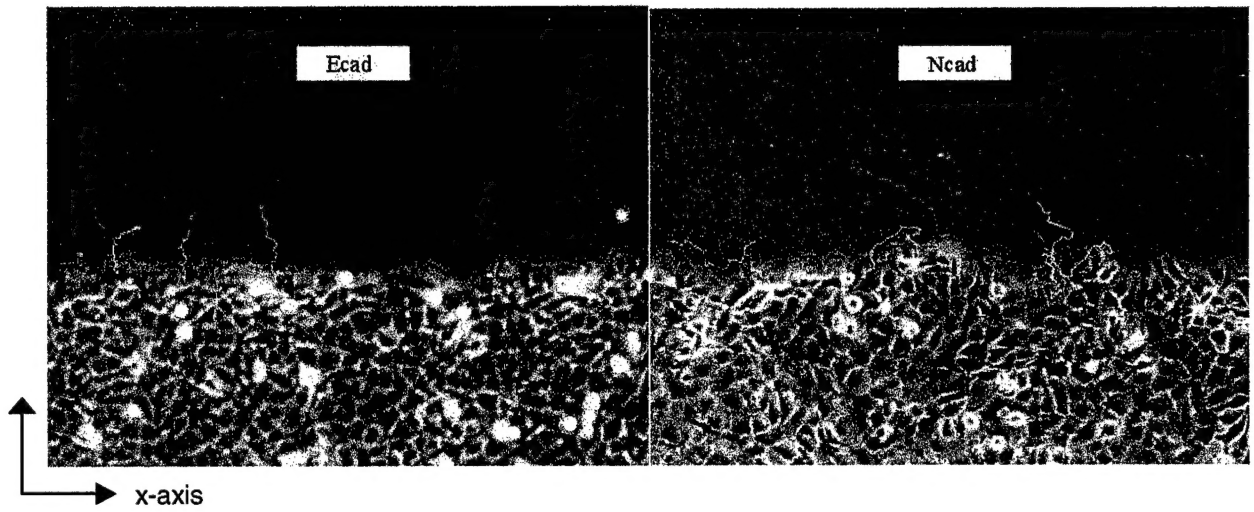
*These results suggested that the key region of E-cadherin includes the transmembrane domain and a segment of the cytoplasmic domain. Furthermore, these results show that the transmembrane and cytoplasmic segments of N-cadherin each permit suppression when combined with E-cadherin, but not when combined with each other! While these findings seem almost self-contradictory, it should be emphasized that these results were consistently obtained with several independently derived cell lines.*

*One interpretation of these unusual findings is that some unidentified component binds to the N/N region and prevents N-cadherin from suppressing motility. This component would not bind to E/E or to the combination of sequences found in E/N or N/E and thus would permit these molecules to suppress. The src-family tyrosine kinase fer binds to N-cadherin sequences adjacent to the plasma membrane, but we found that fer binds equally to E-cadherin and non-suppressing E/N chimeric cadherins.*

***(Year 4)** To explore the hypothesis that N-cadherin is able to suppress motility, but that this ability is masked by the binding of some component near the transmembrane/cytoplasmic domain junction, we have deleted the 9 amino acids just beyond the transmembrane domain and replaced them with the 9 amino acid HA tag. We are currently producing MDA-MB-435 variant cells that express this mutant N-cadherin. We predict that this N-cad mutant will be able to suppress motility.*

*For the first three years of this project, we used the wound-filling assay to quantify movement of cells transfected with various cadherins. This assay is widely used (Etienne-Manneville and Hall, 2001; Kulkarni et al., 2000; Nobes and Hall, 1999) and is generally scored qualitatively or is quantified by computing the distance moved into the wounded area by individual cells or by the edge of the cell monolayer. After considerable experience, we found consistent differences between E- and N-cadherin expressing cells in "appearance" of the monolayer edge, but variable results if assessed by quantitative measures of movement into the monolayer. We used the "appearance" assay to characterize the ability of different chimeric cadherins to suppress motility, summarized in the previous table.*

*Because these differences in appearance were not always reflected in measurements of the extent of wound filling, however, we began quantitatively analyzing our digital time-lapse recordings of the movement of cells in intact monolayers and in the wound-filling paradigm (Objective1, task3). By tracking the movement of individual cells (see figure below) and measuring total movement and movement in the x- and y-axis directions, we found reproducible differences between the movement of cells expressing E- vs. N-cadherin.*




*Each picture shows the edge of the monolayer 60 minutes after wounding and tracks that show the path of individual cells over the next 14 hours, sampled at 15 min intervals. The tracks shown in these pictures suggest that the cells expressing E-cadherin generally tend to move in a y-direction, i.e., to fill in the wound with little lateral (x-axis) movement. N-cadherin expressing cells also fill the wound through y-axis directed movement, but these tracks suggest that the cells expressing N-cadherin show considerably greater x-axis directed movement than the E-cadherin expressing cells.*

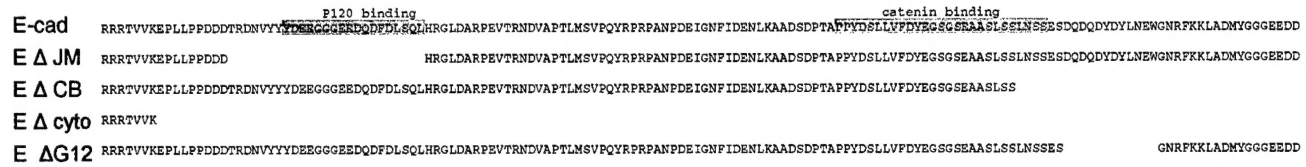
*This quantitative analysis was extended to key chimeric and deletion constructs. The tracks of individual cells were recorded, as described above, and the distances moved in the x-axis, in the y-axis and in total were computed using Metamorph software. The average distance moved (in microns) by 16 cells from each of 5 transfected MDA-MB-435 cell lines is shown in the table below.*

| | puro | Ecad | Ncad | ENN | NEE |
|---|---|---|---|---|---|
| X axis | 153.1 | 84.2 | 214.3 | 161.0 | 88.0 |
| Y axis | 151.0 | 130.8 | 196.4 | 227.0 | 112.5 |
| Total track length | 249.2 | 180.4 | 329.5 | 309.5 | 165.3 |

*Pairwise multiple comparison showed that the average motilities in the x-axis of the puro, Ncad and ENN cells were statistically significantly higher than the average motilities in the x-axis of Ecad and NEE at the significance level of alpha=0.05. These results confirmed the conclusions we had drawn from the subjective "appearance" of the monolayer edge, but placed these conclusions on a sound, statistically significant footing.*

*We also examined the movement of cells that were not at the edge of the wound, but rather several cells "back", well within an intact monolayer. Unexpectedly, the movement of these cells also differed depending on whether E- or N-cadherin was expressed. Further, time-lapse analyses of intact monolayers confirmed that E-cadherin expressing cells were not motile and generally maintained nearest-neighbor relationships, while N-cadherin expressing cells were motile, breaking cell contacts and moving within the monolayer. These findings strongly suggest that the motility differences we observe in the wound-filling assay are intrinsic and not stimulated by factors resulting from production of the wound.*

*To complement our use of chimeric cadherin molecules to define the regions essential for suppressing motility, we also investigated the effects of various E-cad deletion mutants on motility of MDA-MB-435 cells (Objective 1, task 2). The four deletion constructs that we used are diagrammed below. We obtained the ΔJM and ΔCB constructs from M. Takeichi (Nagafuchi and Takeichi, 1988), and we produced the Δcyto and ΔG12 contructs using PCR.*




The figure shows the E-cadherin cytoplasmic domain with the p120 binding site and the catenin-binding site shaded in red. The four cytoplasmic domain deletion mutants that were analyzed are indicated below.

*Our experiments with the ΔG12 mutant were done in collaboration with Ted Meigs and Pat Casey at Duke University. They found that the G12 subfamily of heterotrimeric G proteins, Gα12 and Gα13, interact with the cytoplasmic domain of cadherins, both E and N, causing the release of β-catenin(Meigs et al., 2001). They subsequently mapped the Gα12 binding site to 11 amino acids near the carboxyl end of the cadherin protein(Kaplan et al., 2001). Expression of an activated form of Gα12 in E-cad-expressing MDA-MB-435 cells reverses adhesion and suppression of motility (Meigs et al., 2002). We also produced a deletion of the Gα12 binding site and a permanently transfected MDA-MB-435 line expressing this mutant. This mutation did not affect E-cad's ability to mediate aggregation.*

*The effect of all 4 deletions on motility is shown in the table at right. Surprisingly, deletion of the juxtamembrane domain had no effect on suppression. Deletion of the catenin-binding domain, the Gα12 binding site and the whole cytoplasmic domain abolished the ability of E-cadherin to suppress motility, suggesting that β-catenin binding is essential for suppression. These results are quite different from the results we obtained from similar experiments in a different cell line, the astrocyte-like WC-5 cells(Chen et al., 1997). Clearly cadherin signaling is strongly affected by cell-type specific factors. The WC-5 cells harbor a temperature sensitive Rous sarcoma virus, and thus have a high level of tyrosine kinase activity, which might explain the results.*

| Cadherin | Suppression of motility |
|---|---|
| puro | No |
| E-cad | Yes |
| E Δ JM | Yes |
| E Δ CB | No |
| E Δ cyto | No |
| E ΔG12 | No |

***(Year 4)*** *Although our earlier results indicated that the juxtamembrane domain, now known to include the binding site for p120, is not involved in suppression of motility, recent studies from other laboratories (Anastasiadis et al., 2000; Anastasiadis and Reynolds, 2000; Grosheva et al., 2001; Noren et al., 2000) have suggested a key role for p120 in regulating motility. These labs showed that overexpression of p120 induces a dendritic-morphology and, in some cells analyzed, an increase in cell movement. These changes were shown to result from inhibition of rhoA and activation of rac and cdc42 by p120. All three groups showed that p120 has these effects when free in the cytosol, but does not affect small GTPase activity when it is bound to E-cadherin and, therefore localized at the plasma membrane. Based on these observations, all three groups have proposed that loss of E-cadherin promotes cell motility and invasion of tumors by releasing p120 from the plasma membrane.*

*By analyzing the distribution of p120 in MDA-MB-435 cells transfected with various cadherin constructs, we showed clear discordances between suppression and p120 distribution (see table). These results strongly suggest that regulating the cellular distribution of p120 is not the means by which cadherins suppress motility. One possible explanation for the discrepancy between our findings and those of (Anastasiadis et al., 2000; Anastasiadis and Reynolds, 2000; Grosheva et al., 2001; Noren et al., 2000), however, is that the MDA-MB-435 cells differ from the CHO, MDCK, and 3T3 cells analyzed by the other workers in being unresponsive to p120. We verified, however, that overexpression of p120 induces a dendritic morphology in MDA-MB-435 cells (not shown), as in the other cell types. These results strongly indicate, therefore, that at physiological levels of expression, subcellular localization of p120 does not regulate cell motility.*

| Cadherin | p120 localization | Predicted effect on motility | Observed effect on motility |
|---|---|---|---|
| puro | Cytoplasmic | Motile | Motile |
| E-cad | Membrane | Suppressed | Suppressed |
| N-cad | Memb/cyto | Suppressed | Motile |
| E Δ JM | Cyto/Memb | Motile | Suppressed |
| E Δ CB | Memb/cyto | Suppressed | Motile |

**Technical Objective 2** To determine whether an intact juxtamembrane domain is required for E-cadherin-induced tyrosine phosphorylation.

***(Previously reported)*** *In light of earlier findings, this objective was modified to test whether the critical region of E-cadherin (the transmembrane/juxtamembrane region defined as required for suppression) is necessary for initiating a tyrosine phosphorylation cascade. We found evidence that E-cadherin-mediated contact initiates several parallel cascades of tyrosine phosphorylation. One pathway involves activation of the EphA2 receptor tyrosine kinase and another pathway involves transient activation of the EGF Receptor. By comparing receptor activation with suppression of motility in the panel of cell lines expressing different chimeric cadherins, we were able to prove that neither of these pathways is required for suppression of motility.*

**Technical Objective 3** To identify factors that interact with the juxtamembrane domain of E–cadherin.

***(Previously reported)*** *We confirmed the observations of others that EGFR interacts with E-cadherin, and showed for the first time that the interaction is via their extracellular domains. However, that there was no correlation between suppression of motility and activation of EGFR, so this protein is not required for suppression of motility by E-cadherin. We also began to use specific immunoprecipitation of E-cadherin, followed by 2-dimensional gel electrophoresis to isolate associated proteins and then identify them by tryptic digestion and mass spectrometry analysis.*

***(Year 4)*** *Initial attempts resulted in identification of metavinculin, an alternatively spliced form of vinculin, as an E-cadherin associated protein. Although not previously reported, this was not surprising, as vinculin is known to be cadherin-associated. Subsequent attempts to identify additional proteins were bedeviled by problems with the Mass Spectrometry Core Facilities here at the University of Cincinnati and by variability in the extraction and immunoprecipitation procedures. These problems are being resolved and we expect to identify additional proteins in the near future.*

## KEY RESEARCH ACCOMPLISHMENTS

- Demonstrated that E- and N-cadherin differ in ability to suppress movement of mammary epithelial cells.
- Developed a new assay for quantitative analysis of the effect of cadherins on cell movement. Using this assay, we showed that E-cadherin, but not N-cadherin, suppresses the movement of cells in intact monolayers.
- Determined that the difference between E-cadherin and N-cadherin in ability to suppress motility is localized to the transmembrane domain and a small segment of the cytoplasmic domain.
- Demonstrated that an intact catenin-binding domain is necessary for E-cadherin to suppress motility.
- Showed that cadherin-mediated cell-cell adhesion is required for activation of EphA2 by its ephrin ligands and that this activation reduces focal adhesions, but is not necessary for suppression of cell movement.
- Showed that E-cadherin regulates activation of EGFR by ligand and does so by interactions via the extracellular domains of the two molecules. This activation reduces focal adhesions but, again, is not required for suppression of cell motility.
- We found that E-cadherin does not suppress motility of MDA-MB-435 cells by sequestering p120, as proposed by others.

## REPORTABLE OUTCOMES

Publications:

Zantek, N.D., Azimi, M., Hein, P., Fedor-Chaiken, M., Brackenbury, R., and M.S. Kinch. (1999). E-cadherin regulates the function of the EphA2 receptor tyrosine kinase. *Cell Growth and Differentiation* **10**:629-638.

Meigs, T.E., Fedor-Chaiken, M., Kaplan, D.D., Brackenbury, R., and P.J. Casey. (2002). Gα12 and Gα 13 negatively regulate the adhesive functions of cadherin. *J. Biol. Chem.* **277**:24594-24600.

Hein, P., Chaiken, M., Stewart, J.C., Brackenbury, R., and M. S. Kinch (2002). E-cadherin binding regulates EGF receptor activation. *J. Cell Science*, currently in revision.

Meeting Abstract:

Fedor-Chaiken, M. and R. Brackenbury. (2000). E-cadherin cytoplasmic tail is required to suppress breast cancer cell motility. Era of Hope meeting June 8-11, 2000.

New Cell Lines

We have produced variants of MDA-MB-435 and MDA-MB-231 cells transfected with expression vectors encoding full-length mouse E- and N-cadherins and three E-cadherin deletion mutants (ΔJM, ΔCB and Δcyto).

In addition we have made MDA-MB-435 variants transfected with six E/N chimeric cadherins (ENN, NEE, EEN, NNE, ENE and NEN) and an additional E-cadherin deletion mutant (ΔG12).

## CONCLUSIONS

Cell-cell contact mediated by E-cadherin suppresses movement of mammary epithelial cells. We have demonstrated that it is not the adhesive activity, but more likely a signaling activity of E-cadherin that regulates cell movement. We showed that N-cadherin does not suppress motility and used the difference between these two molecules to define the region of E-cadherin that is required. The results suggest that a region encompassing the transmembrane segment and a small portion of the cytoplasmic domain is crucial. We are currently working to identify proteins that interact with this region, which may be useful diagnostic or therapeutic targets for invasive tumors.

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# E-Cadherin Regulates the Function of the EphA2 Receptor Tyrosine Kinase[1]

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## Abstract

**EphA2 is a member of the Eph family of receptor tyrosine kinases, which are increasingly understood to play critical roles in disease and development. We report here the regulation of EphA2 by E-cadherin. In nonneoplastic epithelia, EphA2 was tyrosine-phosphorylated and localized to sites of cell-cell contact. These properties required the proper expression and functioning of E-cadherin. In breast cancer cells that lack E-cadherin, the phosphotyrosine content of EphA2 was decreased, and EphA2 was redistributed into membrane ruffles. Expression of E-cadherin in metastatic cells restored a more normal pattern of EphA2 phosphorylation and localization. Activation of EphA2, either by E-cadherin expression or antibody-mediated aggregation, decreased cell-extracellular matrix adhesion and cell growth. Altogether, this demonstrates that EphA2 function is dependent on E-cadherin and suggests that loss of E-cadherin function may alter neoplastic cell growth and adhesion via effects on EphA2.**

## Introduction

Protein tyrosine phosphorylation generates the powerful signals necessary for the growth, migration, and invasion of normal and malignant cells (1). A number of tyrosine kinases have been linked with cancer progression (2), and increased tyrosine kinase activity is an accurate marker of cancer progression (3, 4). EphA2 (epithelial cell kinase) is a $M_r$ 130,000 member of the Eph family of receptor tyrosine kinases (5), which interact with cell-bound ligands known as ephrins (1, 6, 7). Whereas EphA2 and most other Eph kinases are expressed and well studied in the developing embryo (8), in the adult, EphA2 is expressed predominantly in epithelial tissues (5). The function of EphA2 is not known, but it has been suggested to regulate proliferation, differentiation, and barrier function of colonic epithelium (9); stimulate angiogenesis (10); and regulate neuron survival (11). Little is known of EphA2's role in cancer, although recent studies demonstrate EphA2 expression in human melanomas (12), colon cancers (9), and some oncogene-induced murine mammary tumors (13).

There is much interest in how tyrosine kinases like EphA2 regulate cell growth and differentiation. One often unappreciated mechanistic hint is the observation that substrates of tyrosine kinases are found almost exclusively within sites of cellular adhesion (14). In epithelial cells, for example, tyrosine-phosphorylated proteins are predominantly located in E-cadherin-associated adherens junctions (14, 15). E-cadherin mediates calcium-dependent cell-cell adhesions through homophillic interactions with E-cadherin on apposing cells (16, 17). In cancer cells, E-cadherin function is frequently destabilized, either by loss of E-cadherin expression (18) or by disruption of linkages between E-cadherin and the actin cytoskeleton (19–23). Restoration of E-cadherin function, either by E-cadherin transfection (24, 25) or treatment with pharmacological reagents (21), is sufficient to block cancer cell growth and induce epithelial differentiation. However, the mechanisms by which E-cadherin imparts these tumor suppressor functions are largely unknown. Whereas E-cadherin-mediated stabilization of cell-cell contacts undoubtedly is involved, there is recent evidence that E-cadherin also generates intracellular signals that could contribute to tumor suppression (15, 26, 27).

Previous studies by our laboratory have linked E-cadherin with signaling by tyrosine phosphorylation. E-cadherin aggregation into assembling adherens junctions initiates a signaling cascade involving tyrosine phosphorylation that may contribute to E-cadherin's tumor suppressor function (28). In addition, we have demonstrated that transformed epithelial cells have elevated levels of tyrosine phosphorylation that destabilize E-cadherin function (21). To identify tyrosine kinases and their substrates in breast cancer, we recently generated monoclonal antibodies that are specific for tyrosine-phosphorylated proteins in Ras-transformed breast epithelial cells (15). Using these antibodies, we identified the EphA2 tyrosine kinase as a protein that is tyrosine-phosphorylated upon E-cadherin-mediated adhesion. We also show that E-cadherin regulates the functioning of EphA2.

## Results

**Regulation of EphA2 Expression in Breast Cancer Cells.** We measured EphA2 expression levels in breast epithelial cell lines derived from nonneoplastic epithelia (*e.g.*, MCF-

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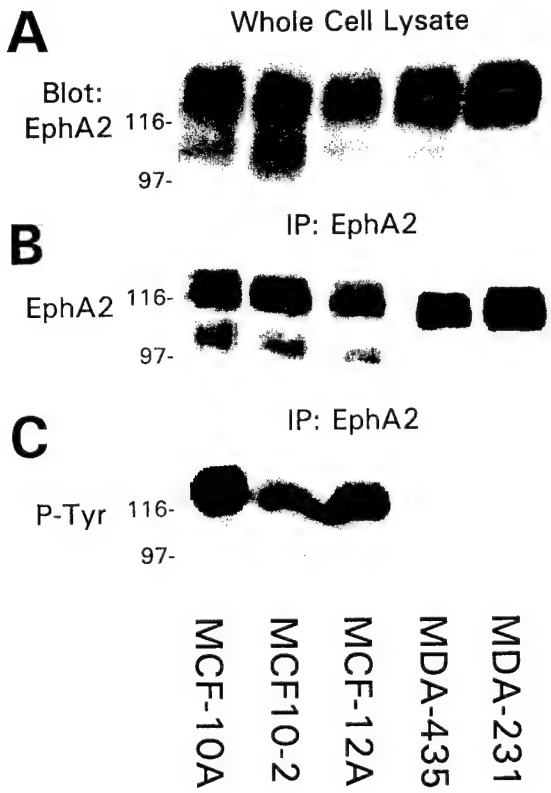


*Fig. 1.* Decreased EphA2 phosphorylation in metastases. EphA2 from whole cell lysates (*A*) or immunoprecipitated from monolayers of nonneoplastic (MCF-10A, MCF10-2, and MCF-12A) and metastatic (MDA-MB-231 and MDA-MB-435) breast cancer cell lines (*B*) was resolved by SDS-PAGE and Western blot analysis performed with EphA2 antibodies. *C*, the blot from *B* was stripped and reprobed with phosphotyrosine-specific (PY20) antibodies. Note the absence of tyrosine-phosphorylated EphA2 in metastatic breast cancer cells.

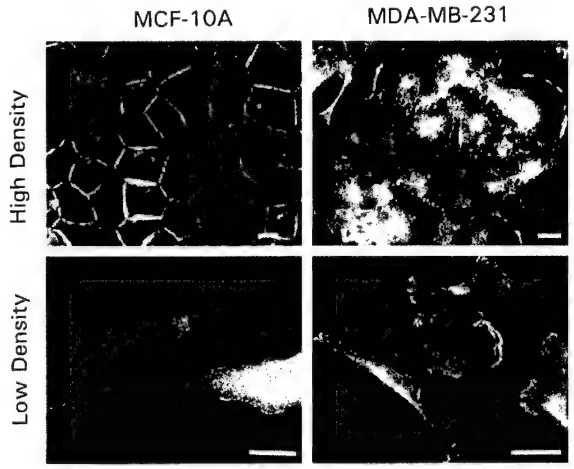


*Fig. 2.* Altered EphA2 localization in metastatic cancer cells. The subcellular distribution of EphA2 in nontransformed mammary epithelial cells (MCF-10A) and metastatic breast cancer cells (MDA-MB-231) was assessed by immunostaining with EphA2-specific antibodies. The cells were plated at either high (*top*) or low (*bottom*) cell density to emphasize the localization of EphA2 within cell-cell contacts or membrane ruffles of nontransformed or invasive cells, respectively. *Scale bars*, 10 $\mu$m.

10A, MCF-12A, and MCF10-2; Refs. 29 and 30) and metastatic breast cancer (*e.g.*, MDA-MB-231 and MDA-MB-435; Refs. 31 and 32). EphA2 was found to be expressed in nontransformed mammary epithelial and metastatic breast cancer cell lines tested (Fig. 1*A* and data not shown), with 2–5-fold more EphA2 in neoplastic cells, as determined by Western blot analysis using multiple EphA2 antibodies and by Northern blot analysis (data not shown).

Despite its overexpression, EphA2 in metastatic cells displayed a much-reduced phosphotyrosine content. For these studies, EphA2 was immunoprecipitated from confluent monolayers of either nonneoplastic or metastatic cells and Western blot analysis performed with phosphotyrosine specific antibodies. This revealed prominent phosphorylation of EphA2 in nonneoplastic cells, whereas the EphA2 from metastatic cells was not tyrosine-phosphorylated (Fig. 1*C*). The decreased phosphotyrosine content was confirmed using different EphA2 antibodies (D7, B2D6, and rabbit polyclonal antibodies) for immunoprecipitation and with multiple phosphotyrosine antibodies (PY20, 4G10, and rabbit polyclonal antibodies) for Western blot analysis (data not shown). Decreased EphA2 phosphorylation was also observed in other metastatic breast cancer cell lines as well as invasive tumor cell lines derived from colon, pancreatic, ovarian, and lung cancers (data not shown).

Further comparison of EphA2 in nonneoplastic and metastatic cells revealed other changes in EphA2 distribution and function. Immunofluorescence staining with EphA2-specific antibodies revealed that EphA2 in nonneoplastic cells was mostly found within sites of cell-cell contact (Fig. 2), with little staining of membrane that was not in contact with neighboring cells. In contrast, EphA2 in metastatic cells was absent from sites of cell-cell contacts. Instead, the EphA2 in these cells was either diffusely distributed or enriched within membrane ruffles at the leading edge of migrating cells. The enrichment within membrane ruffles was confirmed by colocalization of EphA2 with f-actin (data not shown). This localization within membrane ruffles was not observed in nontransformed epithelia, even at low cell density. These differences in subcellular distribution were confirmed using three different EphA2-specific antibodies (D7, B2D6, and rabbit polyclonal antibodies). The correlation between EphA2 localization and phosphotyrosine content forms the basis for much of the remainder of this study.

**EphA2 Enzymatic Activity in Metastatic Cells.** Tyrosine phosphorylation of a kinase often regulates enzymatic activity. To test the effect of differences in EphA2 phosphorylation on kinase activity, we measured EphA2 autophosphorylation by using *in vitro* kinase assays with immunoprecipitated material (Fig. 3). Despite the low phosphotyrosine content of EphA2 in metastatic cells, this EphA2 demonstrated enzymatic activity that was comparable with or higher than the activity of EphA2 isolated from nonneoplastic cells. This activity was unaffected by the basal phosphotyrosine content of EphA2 because unlabeled phosphate was rapidly exchanged with labeled phosphate during the autophosphorylation assays as described previously (33, 34). KOH treatment of the membranes prior to autoradiography did not

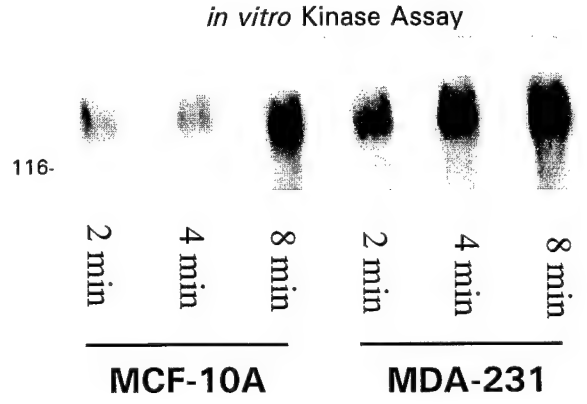


*Fig. 3.* EphA2 enzymatic activity. The enzymatic activity of EphA2 was measured using an *in vitro* autophosphorylation assay. At the times shown, the *in vitro* reaction was terminated and resolved by SDS-PAGE. The blot shown was treated with KOH to hydrolyze phosphoserine and phosphothreonine prior to autoradiography. After several half-lives, Western blot analysis was performed with EphA2 antibodies to confirm equal sample loading (data not shown).

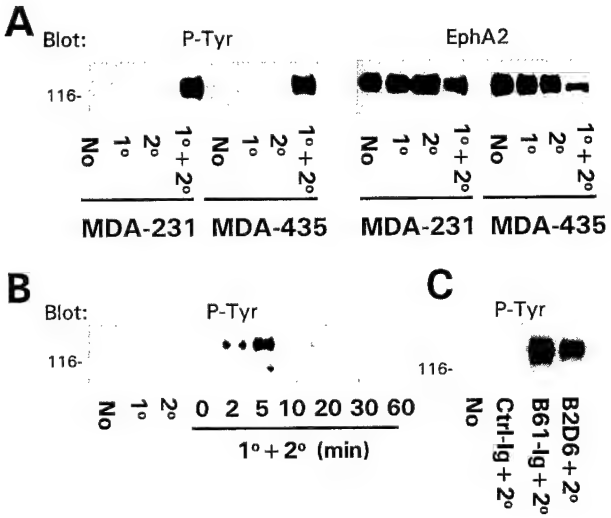


*Fig. 4.* Antibody-mediated aggregation induces EphA2 phosphorylation in metastatic cells. *A*, immunoprecipitated EphA2 was subjected to Western blot analysis with phosphotyrosine antibodies (PY20) following aggregation of cell surface EphA2 for 5 min at 37°C with specific primary and secondary antibodies (*1°+2°*). Note that simple engagement of anti-EphA2 (*1°*) or antimouse (*2°*) alone was insufficient to induce tyrosine phosphorylation above basal levels (*No*). The blot was then stripped and reprobed with EphA2 antibodies as a loading control. *B*, the time course of EphA2 phosphorylation was measured after cross-linking (*1°+2°*) EphA2 in MDA-MB-231 cells for 0–60 min by Western blot analysis of immunoprecipitated EphA2 with phosphotyrosine-specific antibodies (PY20). *C*, EphA2 was aggregated using a soluble ligand fusion protein (B61-IgG). A control fusion protein (Ctrl-IgG) served as a negative control, and B2D6-mediated aggregation served as a positive control for activation.

significantly reduce the level of phosphorylation, indicating that the observed enzymatic activity represented mostly phosphorylation on tyrosine residues. It is also notable that the phosphotyrosine content of EphA2 (Fig. 1*B*) was not predictive of its enzymatic activity (Fig. 3).

**Receptor Aggregation Induces EphA2 Tyrosine Phosphorylation in Metastatic Cells.** EphA2 in neoplastic cells retained the capacity to become activated. For example, EphA2 tyrosine phosphorylation was induced by aggregation of EphA2 with a soluble form of ephrin-A (B61-IgG, a chimera of the EphrinA1 extracellular domain fused to immunoglobulin heavy chain; also known as a "ligand-body"; Refs. 10 and 35; Fig. 4*C*). In contrast, a control chimera (Ctrl-IgG) did not alter EphA2 phosphorylation. Clustering EphA2 at the cell surface with specific antibodies (EK166B or B2D6) also induced levels of EphA2 activation that were comparable with that nonneoplastic cells (Fig. 4*A*). Receptor aggregation, not simply antibody binding, was necessary for EphA2 phosphorylation as incubation with anti-EphA2 (Fig. 4, *1°*) alone did not increase EphA2 phosphorylation relative to matched controls. This effect was specific for EphA2 as neither secondary (Fig. 4, *2°*) antibodies alone or clustering of isotype-matched control antibodies (which recognize an inaccessible cytoplasmic epitope on EphA2) did not induce tyrosine phosphorylation of EphA2 (data not shown). Analysis of the timing of EphA2 phosphorylation revealed EphA2 phosphorylation within 2 min after cross-linking, with optimal phosphorylation detected after 5 min (Fig. 4*B*).

**E-Cadherin Regulates EphA2 in Nontransformed Epithelia.** Tyrosine phosphorylation of EphA2 correlates with its localization within sites of cell-cell contact. Because Eph receptors become activated by ligands that are attached to the surface of neighboring cells (36), we reasoned that stable cell-cell adhesions might be necessary for EphA2 activation. Adhesions mediated by E-cadherin generate the most stable interactions between epithelial cells (16), and we noted that EphA2 was not phosphorylated and was absent from intercellular contacts in cells lacking E-cadherin. These include metastatic cancer cells as well as nontransformed fibroblasts (*e.g.*, NIH 3T3, REF-52, and C3H10T½) and myoepithelial cells (HBL-100; data not shown). We, therefore, tested whether E-cadherin might regulate EphA2 phosphorylation.

Because both EphA2 and E-cadherin are found at sites of cell-cell contact, we first examined whether the two proteins colocalize using two-color immunofluorescence microscopy. This revealed an overlapping distribution of EphA2 and E-cadherin along the lateral membranes of epithelial cells and, specifically, within sites of cell-cell contact (Fig. 5). Vertical sectioning by confocal microscopy confirmed colocalization of E-cadherin and EphA2 within sites of cell-cell contact (data not shown).

To test whether the colocalization of EphA2 and E-cadherin might indicate a functional link between the two proteins, we disrupted calcium-dependent E-cadherin-mediated adhesion by supplementing the cell culture medium with 4 mM EGTA, a calcium-chelating agent. EGTA treatment caused EphA2 dephosphorylation (Fig. 6*A*) and induced either a diffuse or membrane ruffle pattern of staining (Fig. 6*C*), which was reminiscent of EphA2 in metastatic cells. Subsequent restoration of normal levels of extracellular calcium restored normal levels of EphA2 phosphorylation and cell-cell localization within 5 min (Fig. 6, *A* and *C*).

Although results with EGTA-treated samples implicate cell-cell adhesion with the control of EphA2 phosphorylation

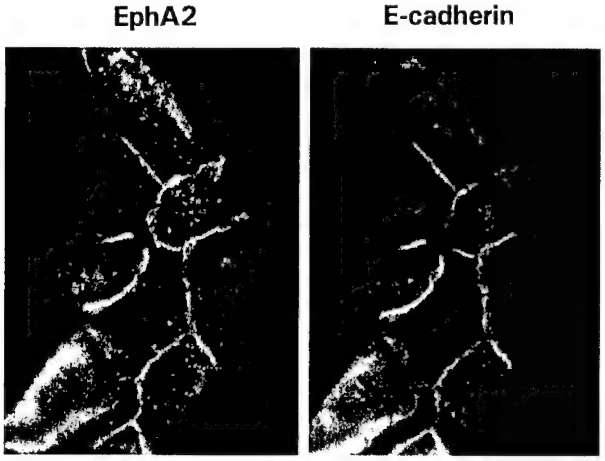


*Fig. 5.* Colocalization of EphA2 and E-cadherin. The subcellular distribution of EphA2 (*left*) and E-cadherin (*right*) was evaluated in MCF-10A cells using two-color immunofluorescence microscopy. Note the overlapping distribution of EphA2 and E-cadherin within sites of intercellular junctions.

and subcellular localization, we sought to determine whether E-cadherin contributed to this regulation. For this, we supplemented the cell culture medium with function-blocking E-cadherin antibodies and peptides (DECMA-1 antibodies or HAV peptides; Refs. 37 and 38). When inhibitors of E-cadherin function were added to the medium concomitant with the restoration of extracellular calcium, EphA2 did not become tyrosine-phosphorylated (Fig. 7*A*) and remained diffuse or present within membrane ruffles (Fig. 7*C*). In contrast, isotype-matched control antibodies and scrambled peptides did not prevent EphA2 phosphorylation or localization within intercellular junctions. Specific inhibition of E-cadherin with these inhibitors also blocked EphA2 phosphorylation and cell-cell localization upon treatment of confluent cell monolayers (data not shown), thus confirming that EphA2 phosphorylation and localization are sensitive to the functioning of E-cadherin.

**EphA2 Is Responsive to E-Cadherin Expression in Metastatic Cells.** To examine further the link between EphA2 and E-cadherin, we transfected MDA-MB-231 cells with E-cadherin (231-E-cad) and selected for levels of E-cadherin expression that were equivalent to MCF-10A cells. As controls, we transfected cells with empty vector (231-neo). EphA2 in 231-neo was not phosphorylated and was enriched within membrane ruffles (Fig. 8). In contrast, the EphA2 in 231-E-cad redistributed into sites of cell-cell contacts and had levels of phosphotyrosine that were comparable with that of MCF-10A cells (Fig. 9*A*). These changes in EphA2 phosphorylation and localization increased with cell density (data not shown), consistent with an idea that E-cadherin function regulates EphA2 phosphorylation and localization.

**EphA2 Regulates Cell Adhesion and Proliferation.** Microscopic analysis revealed that E-cadherin expression altered the adhesive profile of MDA-MB-231 cells (Fig. 8). Whereas parental and 231-neo cells were mesenchymal in appearance and readily grew atop one another, the E-cadherin-transfected cells had more prominent cell-cell adhesions and grew as single-cell monolayers. Analysis of cell-ECM[3] attachments by staining with paxillin-specific antibodies revealed numerous focal adhesions in control MDA-MB-231 cells, whereas 231-E-cad cells had fewer focal adhesions. The decrease in focal adhesions was most prominent in 231-E-cad cells within colonies (Fig. 8, *bottom right*), whereas individual cells had focal adhesions that were comparable with controls (data not shown).

EphA2 activation contributes to the decreased cell-ECM adhesion. To activate EphA2 in MDA-MB-231 cells, we aggregated EphA2 at the cell surface with specific antibodies (as described above) and found that this caused a rapid loss of focal adhesions within 5 min. This was confirmed by paxillin staining (Fig. 10) and by interference reflection microscopy (data not shown). Similar results were obtained in other neoplastic cell lines (data not shown). In contrast, treatment with either primary or secondary antibodies alone did not alter focal adhesions.

Focal adhesions are sites of intracellular signaling that promote cell growth (39, 40). Because EphA2 activation blocks focal adhesions, we questioned whether EphA2 activation would impact cell growth. To test this, we activated EphA2 with specific antibodies or B61-IgG ligand-bodies (as described above). Concomitant with receptor cross-linking, we included BrdUrd in the culture medium and measured DNA synthesis over the following 4 h. As shown in Table 1, EphA2 activation decreased the proliferation in MDA-MB-231 cells (31% reduction; $P < 0.001$), whereas control conditions (primary or secondary antibodies alone and isotype controls) did not change cell growth. The short duration of EphA2 signaling that is induced by antibody aggregation (Fig. 4*B*) likely underestimates EphA2's growth-inhibitory potential. A similar decrease in cell growth was obtained following EphA2 activation in other cell types, including MDA-MB-435 cells (22% reduction; $P < 0.0005$) and MCF-10A cells (16% reduction; $P < 0.01$). For experiments with MCF-10A, we plated cells at low cell density and scored individual cells (to preclude cell-cell contacts that might otherwise activate EphA2).

## Discussion

The major findings of this study are that the localization and phosphorylation of EphA2 in mammary epithelial cells are dependent on E-cadherin-mediated adhesion and that loss of E-cadherin in metastatic tumor cells causes alterations in EphA2 localization and phosphorylation. In addition, we found that experimental induction of EphA2 phosphorylation decreases cell-ECM attachment at focal adhesions and negatively regulates the proliferation of metastatic cells.

**Decreased EphA2 Phosphorylation in Metastatic Cells.** We originally identified EphA2 using antibodies that recognize tyrosine-phosphorylated proteins in Ras-transformed MCF-10A-neoT cells (15). MCF-10A-neoT cells express E-cadherin (21) and, consequently, EphA2 is tyrosine-phosphorylated (data not shown). Notably, EphA2 was tyrosine-

[3] The abbreviations used are: ECM, extracellular matrix; BrdUrd, bromodeoxyuridine.

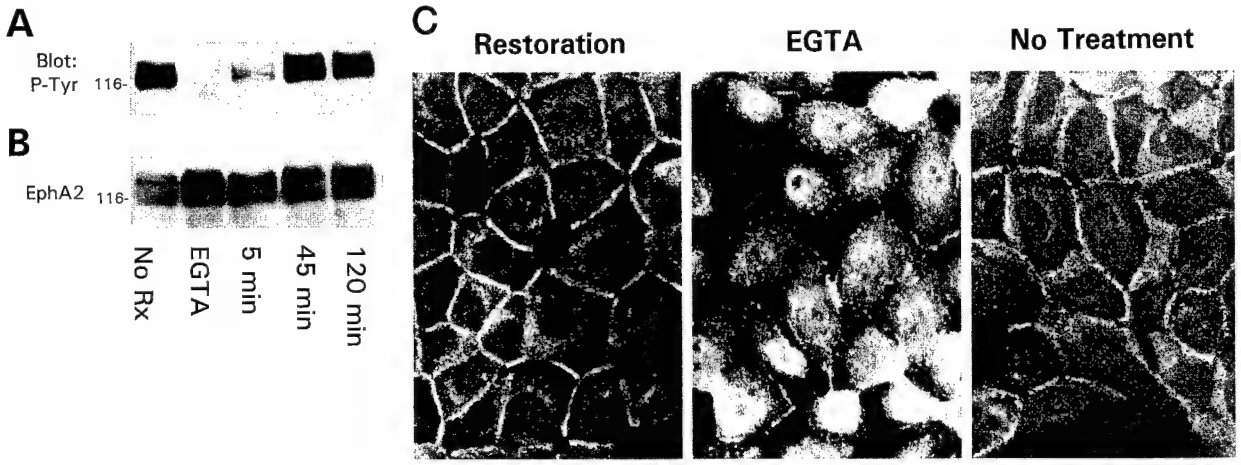


*Fig. 6.* EphA2 phosphorylation and localization require stable E-cadherin adhesions. Stable cell-cell contacts in monolayers of MCF-10A cells were disrupted by the addition of EGTA (4 mM, 30 min, 37°C) to the culture medium. After removal of the EGTA, normal growth medium was returned for 0–120 min. *A*, EphA2 was immunoprecipitated and Western blot analysis performed with phosphotyrosine-specific (PY20) antibodies. *B*, the blot from *A* was stripped and reprobed with EphA2 antibodies as a loading control. *C*, staining with EphA2-specific antibodies assessed changes in the subcellular distribution of EphA2 before and after restoration of cell-cell adhesions.

phosphorylated in nonneoplastic mammary epithelial cell lines but not in metastatic cell lines. In this respect, EphA2 differs from many other tyrosine kinases (*e.g.*, cErbB2, epidermal growth factor receptor, platelet-derived growth factor receptor, and Src), the phosphorylation of which increases in cancer cells (2, 41, 42). For these kinases, phosphorylation elevates tyrosine kinase activity, triggering signal transduction cascades that promote cell proliferation.

The phosphotyrosine content of EphA2 does not relate to its intrinsic enzymatic activity in mammary epithelial cells. *In vitro* assays revealed that, despite its low phosphotyrosine content, the enzymatic activity of EphA2 in metastatic cells is comparable with or increased over the activity of phosphorylated EphA2 in nonneoplastic epithelial cells. This is consistent with evidence that the phosphorylation of EphB2 also has little effect on its kinase activity (43). Our results suggest that, rather than controlling enzymatic activity, the phosphotyrosine content of EphA2 might influence the choice or availability of substrates and interacting proteins. In addition, changes in the phosphotyrosine content of EphA2 might provide signals that are independent of EphA2 enzymatic activity, which is supported by recent reports that other Eph kinases (VAB-1 and EphB2) have kinase-independent functions (44, 45). This suggests that protein interactions, localization, phosphotyrosine content, and enzymatic activity all contribute to Eph receptor function.

There are several possible explanations for the loss of EphA2 phosphorylation in metastatic cells. The primary sites of receptor autophosphorylation are not mutated because the sites that become autophosphorylated *in vitro* are the same in nontransformed and neoplastic cells.[4] Consistent with this, EphA2 tyrosine phosphorylation was restored by cross-linking EphA2 with antibodies or by transfection with E-cadherin. Another possible cause for decreased EphA2 phosphorylation could be loss of EphA2 ligands (ephrin-A class molecules). However, our ability to restore EphA2 phosphorylation in E-cadherin-transfected cells appears to exclude this possibility. A third possibility is that the phosphotyrosine content of EphA2 is repressed by an associated tyrosine-phosphatase. Consistent with this, treatment of neoplastic cells with tyrosine-phosphatase inhibitors restores normal levels of EphA2 tyrosine phosphorylation.[5] However, the identities of the phosphatases responsible for this are presently unknown.

**Regulation of EphA2 Activation by E-Cadherin.** We focused on the possibility that decreased stability of cell-cell contacts inhibits tyrosine phosphorylation of EphA2 in metastatic cells. Both Eph family receptor tyrosine kinases and their ephrin ligands are bound to the cell surface (1, 6, 7), so cells must be in close contact to facilitate Eph-ephrin interactions. Little is known, however, about the nature of these contacts and their precise effects on Eph-ephrin interactions.

Because many breast tumors lack E-cadherin and have unstable cell-cell junctions (18, 46), we investigated how expression of E-cadherin affects EphA2 phosphorylation in mammary epithelial cells. We found inhibition of E-cadherin function either by removal of $Ca^{2+}$ or with function-blocking antibodies or peptides reduced EphA2 phosphorylation and caused EphA2 to redistribute into membrane ruffles. Conversely, expression of E-cadherin in MDA-MB-231 cells restored EphA2 phosphorylation and localization to sites of cell-cell contact. The simplest explanation for these results is that E-cadherin stabilizes cell-cell contacts and, thereby, facilitates interactions between EphA2 and its ligands.

At present, there is no evidence for or against a direct interaction between E-cadherin and EphA2. The two proteins are expressed in overlapping patterns. but we have not been able to coimmunoprecipitate EphA2 and E-cadherin.[5] EphA2

[4] M. S. Kinch, unpublished results.

[5] N. D. Zantek, unpublished results.

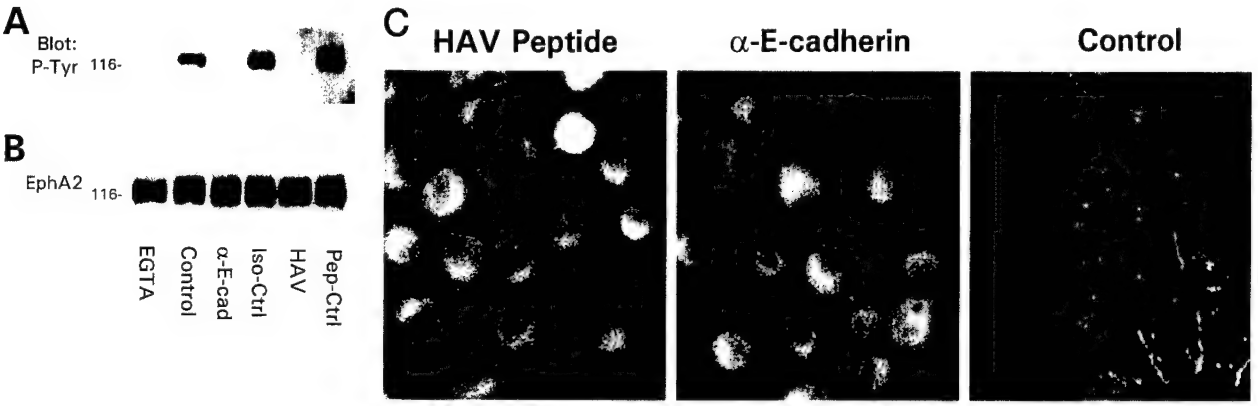


*Fig. 7.* Inhibition of E-cadherin-mediated adhesion. Following treatment of MCF-10A cell monolayers with EGTA, normal medium conditions were restored in the absence (*Control*) or presence of function-blocking E-cadherin antibodies (*α-E-cad*) or peptides (*HAV*). Isotype control antibodies (*Iso-Ctrl*) and scrambled peptides (*Pep-Ctrl*) were included as matched negative controls. *A*, immunoprecipitated EphA2 was subjected to Western blot analysis with phosphotyrosine (PY20) antibodies. *B*, the same blot as in *A* was stripped and reprobed with EphA2 antibodies as a loading control. *C*, EphA2 localization was determined after calcium restoration in the absence (*Control*) or presence of E-cadherin inhibitors.

also does not cocluster with E-cadherin at the cell surface in response to antibody-mediated aggregation of either molecule,[6] which is consistent with our biochemical evidence. We cannot exclude that experimental conditions used for protein extraction dissociate such interactions or that a small fraction of activated EphA2 coclusters with E-cadherin. Direct interaction between the two molecules may not be necessary if E-cadherin primarily serves to stabilize cell-cell contacts and thereby promote interactions between EphA2 and its ligands. Other aspects of E-cadherin function, such as signaling (28), cytoskeletal association (47), and junction formation (16) might also target EphA2 to sites of cell-cell contact.

**EphA2 Regulates Cell-ECM Adhesion and Growth.** An immediate consequence of EphA2 activation is decreased cell-ECM contact at focal adhesions. Focal adhesions are sites of membrane-cytoskeletal interaction that provide anchorage for cell migration and invasion (48). Focal adhesions also play critical roles in signal transduction, where they organize intracellular signals that control cell growth and survival (39, 40). We propose that E-cadherin-mediated stabilization of ligand binding induces EphA2 to block focal adhesions. Consistent with this, it is understood that epithelial cells balance their cell-cell and cell-ECM adhesions and that this is linked with the proper functioning of E-cadherin (49, 50). Individual epithelial cells have more focal adhesions than cells within colonies, whereas cells with decreased E-cadherin function have increased cell-matrix adhesion, regardless of cell density (21). Although the molecular mechanisms responsible for this are unknown, many proteins that interact with Eph kinases regulate cell adhesion or cytoskeletal organization, including the p85 subunit of phosphatidylinositol 3′-kinase, Src, Fyn, and Ras-GAP (35, 51–53).

Focal adhesions initiate signals that promote cell growth, and it follows that loss of these structures may contribute to decreased cell growth following EphA2 activation. By inference, loss of EphA2 activation might contribute to deregulated growth of neoplastic cells by increasing signals from focal adhesions. This would be consistent with evidence that neoplastic cells have increased signaling by focal adhesion proteins (*e.g.*, FAK; Ref. 54). Although EphA2 activation decreases cell growth, the expression pattern of EphA2 does not fit the classic pattern of a tumor suppressor. Most tumor suppressors are inactivated either because of decreased expression or loss of enzymatic activity. In contrast, neoplastic cells express high levels of EphA2, which, although nonphosphorylated, retains comparable levels of enzymatic activity. An alternative explanation is that EphA2 positively regulates cell growth but that this signaling is reduced in nontransformed epithelia. Support for this includes evidence that EphA2 is overexpressed in neoplastic cells and is supported by the fact that other Eph kinases (*e.g.*, EphA1) are oncogenic (55). In this scenario, EphA2 "activation" by E-cadherin or receptor aggregation might decrease EphA2 function, perhaps by reducing EphA2 expression levels. It is intriguing that the lowest levels of EphA2 are found in cells where it is phosphorylated and that ligand-mediated aggregation decreases EphA2 expression levels. A third possibility is that EphA2 functions very differently in normal and neoplastic epithelia. The phosphotyrosine content and subcellular localization of EphA2 differ in normal and neoplastic cells, and either property could alter substrate specificity or availability. Indeed, tyrosine-phosphorylated EphA2 (but not unphosphorylated EphA2) interacts with the phosphatidylinositol 3′-kinase and the SLAP adapter protein (56). SLAP was recently shown to negatively regulate cell growth (57), which is supportive of our evidence that EphA2 also regulates cell proliferation. Future studies will be necessary to define EphA2's role as a positive and/or negative regulator of cell growth and to determine whether these properties differ between normal and neoplastic epithelia.

**Conclusions.** Loss of E-cadherin in carcinomas promotes invasion (18, 58), cell motility (27), and cell proliferation (26). In this study, we have identified the receptor tyrosine kinase EphA2 as one protein that is phosphorylated after cell-cell contact and demonstrated that both the phospho-

[6] M. Fedor-Chaiken and M. S. Kinch, unpublished results.

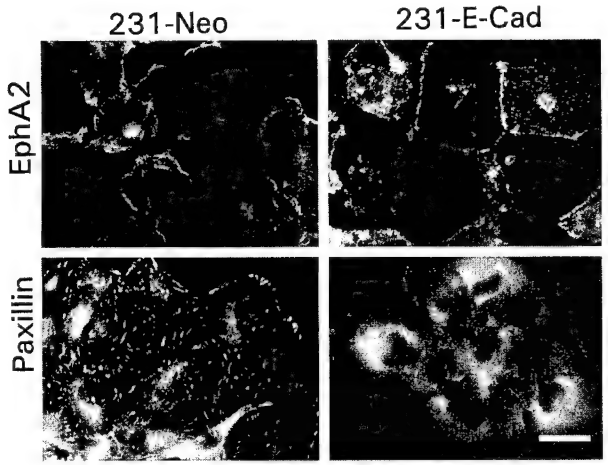


*Fig. 8.* E-Cadherin expression directs EphA2 into cell-cell contacts. The subcellular distribution of EphA2 and paxillin was assessed by immunofluorescence microscopy in control (*231-Neo*) and E-cadherin transfected (*231-E-Cad*) MDA-MB-231 cells. Note that E-cadherin promotes a redistribution of EphA2 into cell-cell contacts and decreases focal adhesions. *Scale bar*, 25 $\mu$m.

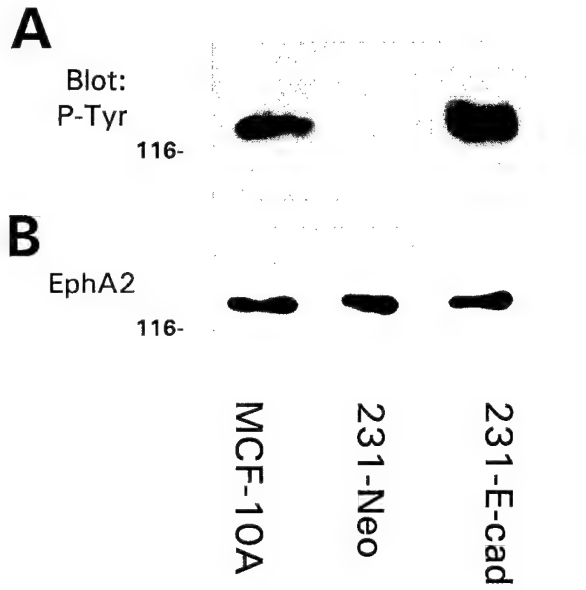


*Fig. 9.* E-cadherin expression restores normal EphA2 function. *A*, the phosphotyrosine content of immunoprecipitated EphA2 was measured by Western blot analysis following transfection of MDA-MB-231 cells with E-cadherin (*231-E-cad*) or a matched vector control (*231-Neo*). MCF-10A was included as a positive control for EphA2 tyrosine phosphorylation. *B*, The blot from *A* was stripped and reprobed with EphA2-specific antibodies as a loading control.

rylation and localization of EphA2 are sensitive to changes in E-cadherin function and expression. We also find that EphA2 activation negatively regulates cell-ECM adhesion and cell growth. These findings raise the possibility that important effects of E-cadherin on tumor cell behavior may occur via effects on EphA2.

## Materials and Methods

**Cell Lines and Antibodies.** Human breast carcinoma cells and nontransformed human mammary epithelial cell lines were cultured as described previously (29, 46). We purchased antibodies specific for E-cadherin (polyclonal antibodies, Transduction Laboratories, Lexington, KY; and DECMA-1, Sigma Chemical Co., St. Louis, MO), phosphotyrosine (PY20, ICN, Costa Mesa, CA; 4G10, Upstate Biotechnology Inc., Lake Placid, NY; and polyclonal antibodies, Transduction Laboratories), and fluorescein-conjugated BrdUrd (Harlan Sera-Lab Ltd., Loughborough, United Kingdom). Monoclonal antibodies specific for EphA2 (clones D7 and B2D6) were produced in the laboratory as described (15) or purchased from Upstate Biotechnology Inc. Rabbit polyclonal antibodies for EphA2 were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). EK166B monoclonal EphA2 antibodies were generously provided by R. Lindberg (Amgen, Thousand Oaks, CA). Paxillin-specific antibodies were obtained from K. Burridge (University of North Carolina, Chapel Hill, NC). To visualize f-actin, we used fluorescein-conjugated phalloidin, purchased from Molecular Probes (Eugene, OR).

**Western Blot Analysis.** Unless noted otherwise, all experiments used confluent cell monolayers that were extracted in a buffer containing 1% Triton X-100 or in RIPA buffer containing 1% Triton X-100, 0.5% sodium deoxycholate, and 0.1% SDS for 6 min on ice, as described previously (21). After protein concentrations were measured by Coomassie Blue staining (Pierce, Rockford, IL) or Bio-Rad $D_C$ Protein Assay (Hercules, CA), equal amounts of protein were resolved by SDS-PAGE and transferred to nitrocellulose (Protran, Schleicher & Schuell, Keene, NH), and Western blot analysis was performed as described previously (21). Antibody binding was detected by enhanced chemiluminescence as recommended by the manufacturer (Pierce). To reprobe, we stripped blots as described previously (21).

**Immunofluorescence and Confocal Microscopy.** Immunostaining was performed as described previously (21). In brief, cells were grown on glass coverslips to visualize individual cells. Cells were observed at both high cell density (~70% confluence) and low cell density (~20% confluence) by seeding $1 \times 10^6$ cells onto either a 3.5- or 10-cm tissue culture plate overnight at 37°C. At high cell density, extensive overlapping of neoplastic cells precludes accurate subcellular visualization. The samples were fixed in 3.7% formaldehyde solution, extracted in 0.5% Triton X-100, and stained. Immunostaining was visualized using rhodamine-conjugated donkey antimouse antibodies (Chemicon, Temecula, CA) and FITC-conjugated donkey antirabbit (Chemicon) and epifluorescence microscopy (model BX60, ×600, Olympus Lake Success, NY) and recorded onto T-Max 400 film (Eastman-Kodak, Rochester, NY). For confocal microscopy, samples were viewed on a Nikon Diaphot 300 outfitted with a Bio-Rad MRC 1024 UV/Vis System and Coherent Innova Enterprise model 622 60-mW output water-cooled lasers.

**Immunoprecipitation.** Immunoprecipitation experiments were performed as described (21) for 1.5 h at 4°C with the appropriate EphA2-specific monoclonal antibodies (D7 or B2D6) and rabbit antimouse (Chemicon) conjugated protein A-Sepharose (Sigma). Immunoprecipitates were washed three times in lysis buffer, resuspended in SDS sample buffer (Tris buffer containing 5% SDS, 3.8% DTT, 25% glycerol, and 0.1% bromphenol blue), and resolved by 10% SDS-PAGE.

***In Vitro* Kinase Assays.** For *in vitro* autophosphorylation assays, immunoprecipitated EphA2 was washed in lysis buffer and incubated in 10 mM PIPES, 3 mM $MnCl_2$, 5 mM PNPP (Sigma 104 phosphatase substrate; Sigma), 1 mM $NaVO_4$, 1 $\mu$M ATP, and 10 $\mu$Ci of [$\gamma$-$^{32}$P]ATP (New England Nuclear, Boston, MA) at 25°C for the times shown. The reactions were terminated by the addition of 5× Laemmli sample buffer at multiple time points before saturation. After resolving samples by 10% SDS-PAGE, the gel was transferred to nitrocellulose (Schleicher & Schuell) or Immobilon P (Pierce), and incorporated material was detected by autoradiography. To hydrolyze phosphoserine/threonine, we treated the membranes with 1 N KOH at 65°C for 1 h and reassessed them by autoradiography. After several half-lives, Western blot analysis was performed to determine EphA2 loading.

**Cross-Linking of EphA2 Receptors.** For antibody cross-linking experiments, cells grown as a monolayer were incubated at 4°C for 20 min with 4 $\mu$g/ml EphA2 antibody (either clone EK166B or B2D6) or purified fusion protein of ephrin-A1 fused to IgG (B61-IgG; Ref. 10). Primary antibody alone, rabbit antimouse IgG alone and control fusion proteins were used as controls. The samples were washed with medium, incubated with 20 $\mu$g/ml rabbit antimouse IgG in conditioned medium at 4°C

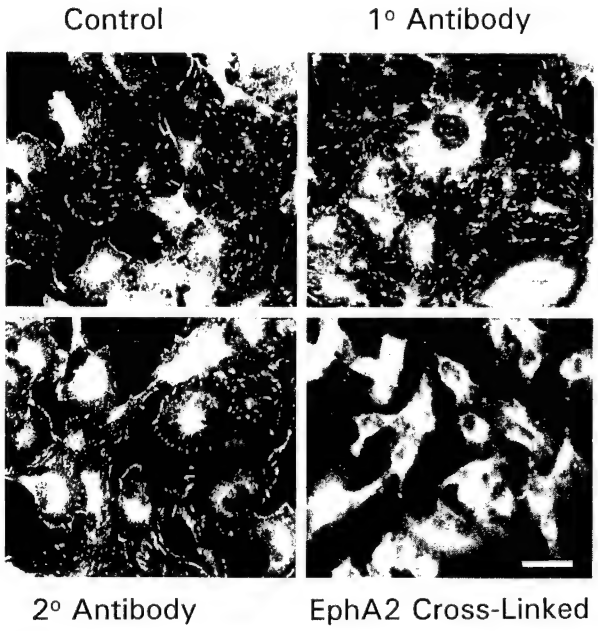


*Fig. 10.* EphA2 activation decreases cell-ECM adhesion. The presence of focal adhesions was assessed by immunostaining for paxillin in MDA-MB-231 cells before and after activation of EphA2 by antibody-mediated aggregation. Note that incubation of cells with either primary (*1°*) or secondary (*2°*) antibodies alone did not alter the presence of focal adhesions, whereas EphA2 aggregation dissipated focal adhesions. *Scale bar*, 25 μm.

*Table 1* EphA2 Activation Inhibits Cell Proliferation[a]

| Cell line | Treatment | % BrdUrd uptake (mean ± SE) | Statistical analysis[b] (*P*) |
|---|---|---|---|
| MDA-MB-231 | Untreated | 43.8 ± 2.0 | |
| | Primary Ab[c] alone | 44.1 ± 2.2 | >0.43 |
| | Secondary Ab alone | 39.7 ± 2.3 | >0.21 |
| | Primary + secondary | 30.4 ± 1.7 | <0.0001 |
| | Control-IgG + secondary | 43.0 ± 2.1 | >0.44 |
| | B61-IgG + secondary | 29.1 ± 3.1 | <0.01[d] |
| MDA-MB-435 | Untreated | 52.8 ± 5.1 | |
| | Primary Ab alone | 52.6 ± 3.4 | >0.25 |
| | Secondary Ab alone | 52.8 ± 6.3 | >0.39 |
| | Primary + secondary | 39.6 ± 3.0 | <0.00005 |
| MCF-10A (low density) | Untreated | 53.6 ± 1.8 | |
| | Primary Ab alone | 53.9 ± 0.8 | >0.43 |
| | Secondary Ab alone | 55.1 ± 0.5 | >0.22 |
| | Primary + secondary | 45.0 ± 1.4 | <0.01 |

[a] BrdUrd uptake into newly synthesized DNA was measured for 4 h after cross-linking of EphA2 at the cell surface with specific antibodies. The data represent at least three independent, double-blinded experiments. Cell growth was determined in at least 100 cells from each experimental and control, and the results shown are compared with DNA synthesis with untreated (untreated) samples. None of the differences between or among individual negative controls (untreated, primary antibody alone, or secondary antibody alone) were significant ($P > 0.05$).
[b] Statistical analyses compared the experimental to untreated for each sample.
[c] Ab, antibody.
[d] For the fusion proteins, there was also a significant difference ($P < 0.02$) between the control and B61 fusion proteins.

for 10 min, and warmed to 37°C for 10 min before extraction and immunoprecipitation. To determine the optimal time for activation, we incubated the plates in the presence of cross-linking antibody at 37°C for 0–120 min.

**EGTA and Antibody Treatments.** "Calcium switch" experiments were performed as described previously (28). Monolayers of MCF-10A cells were grown to ~80% confluence. EGTA was added to growth medium to a final concentration of 4 mM, and the cells were incubated at 37°C for 30 min. The medium was removed, and calcium concentrations restored with normal growth medium. To block E-cadherin function, we supplemented the medium with E-cadherin antibodies (1:100 dilution; DECMA-1; Sigma) or 10 μg/ml peptide corresponding to the E-cadherin HAV sequence (YTLFS*HAV*SSNGN). Controls include isotype control antibodies (rat anti-HA antibody; Boehringer Mannheim, Indianapolis, IN) and matched, scrambled peptides (SGATNSLHNFSVY). The Purdue Laboratory for Macromolecular Structure synthesized peptides. Cells were then incubated for the indicated times at 37°C and extracted for Western blot analysis and immunoprecipitation. Cell monolayers grown on glass coverslips were treated in the same manner and immunostained for EphA2.

**E-Cadherin Expression and Function.** MDA-MB-231 cells were co-transfected with pBATEM2, a mouse E-cadherin expression vector (59) and pSV2neo (60) using FuGENE 6 Transfection Reagent (Boehringer Mannheim), following the manufacturer's instructions. Transfected cells were selected in growth media supplemented with 400 μg/ml G418. Immunostaining and Western blot analysis with specific antibodies confirmed E-cadherin expression.

**Proliferation Assay.** Cells were plated onto glass coverslips and cultured overnight in growth medium. EphA2 antibodies (EK166B or B2D6, extracellular or D7, intracellular) or ligand fusion protein (B61-IgG) were added to the media at 1 μg/ml and incubated at 4°C for 20 min, washed with medium, and incubated with 20 μg/ml rabbit antimouse plus 3 μg/ml BrdUrd at 37°C for 4 h. Cells were fixed in cold methanol for 8 min, extracted with 2 N HCl at 37°C for 30 min and stained with a BrdUrd antibody to indicate proliferating cells and Hoechst dye to label the nuclei of all cells on the coverslip. A minimum of six random fields were selected in a double-blind study, and at least 150 cells were assessed in each sample. Each experiment was repeated at least three times.

**Statistical Methods.** All statistical analyses were performed using the SAS System for Windows, Version 6.12. An ANOVA model was used to compare the percentage of cells that grew in each field, within each specimen, in the control group to the percentage of cells that grew in each field, within each specimen, in the experimental group. Group (control *versus* experimental) was treated as a fixed effect and specimen within each group was treated as a random effect. A normal probability plot of the residuals was used to assess the homogeneity of the variances of the mean percentage cell growth for the control and experimental groups. $P < 0.05$ was considered statistically significant.

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# $G\alpha_{12}$ and $G\alpha_{13}$ Negatively Regulate the Adhesive Functions of Cadherin*

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**Cadherins function to promote adhesion between adjacent cells and play critical roles in such cellular processes as development, tissue maintenance, and tumor suppression. We previously demonstrated that heterotrimeric G proteins of the $G_{12}$ subfamily comprised of $G\alpha_{12}$ and $G\alpha_{13}$ interact with the cytoplasmic domain of cadherins and cause the release of the transcriptional activator β-catenin (Meigs, T. E., Fields, T. A., McKee, D. D., and Casey, P. J. (2001) *Proc. Natl. Acad. Sci. U. S. A.* 98, 519–524). Because of the importance of β-catenin in cadherin-mediated cell-cell adhesion, we examined whether $G_{12}$ subfamily proteins could also regulate cadherin function. The introduction of mutationally activated $G_{12}$ proteins into K562 cells expressing E-cadherin blocked cadherin-mediated cell adhesion in steady-state assays. Also, in breast cancer cells, the introduction of activated $G_{12}$ proteins blocked E-cadherin function in a fast aggregation assay. Aggregation mediated by a mutant cadherin that lacks $G_{12}$ binding ability was not affected by activated $G_{12}$ proteins, indicating a requirement for direct $G_{12}$-cadherin interaction. Furthermore, in wound-filling assays in which ectopic expression of E-cadherin inhibits cell migration, the expression of activated $G_{12}$ proteins reversed the inhibition via a mechanism that was independent of $G_{12}$-mediated Rho activation. These results validate the $G_{12}$-cadherin interaction as a potentially important event in cell biology and suggest novel roles for $G_{12}$ proteins in the regulation of cadherin-mediated developmental events and in the loss of cadherin function that is characteristic of metastatic tumor progression.**

Members of the cadherin family of cell-surface proteins are responsible for the regulation of a wide variety of cellular and multicellular processes within an organism. Classical cadherins are single-pass transmembrane proteins that mediate cell-cell adhesion in a $Ca^{2+}$-dependent manner and are found in nearly all of the solid tissues (1–4). The successful completion of many steps during early embryonic development depends on the ability to regulate cadherin-mediated cell-cell adhesion in a spatial and temporal manner (4, 5). In addition, a growing body of evidence has implicated cadherins as tumor suppressor proteins. For example, malignant tumor cells often exhibit a loss of cadherin function that coincides with the transition to an invasive metastatic state (6–8), and the re-expression of E-cadherin in these cells can suppress their invasive ability (9). The ability of cadherins to bind the transcriptional activator β-catenin at the plasma membrane may contribute to their tumor-suppressive function (10, 11), because unchecked accumulation of free β-catenin in the cytoplasm and nucleus is believed to be a key causative agent in a variety of cancers (12).

We recently reported a surprising connection between cadherins and the $G_{12}$ subfamily of heterotrimeric G proteins that is comprised of $G\alpha_{12}$ and $G\alpha_{13}$ (13). Mutationally activated $G_{12}$ proteins were found to interact with the cytoplasmic domain of cadherins in a fashion that caused a release of β-catenin from cadherin. In subsequent studies, the $G\alpha_{12}$ binding region of E-cadherin was mapped to an 11-residue highly charged domain near the C terminus of the cytoplasmic tail (14). This unexpected connection between heterotrimeric G proteins and the cadherin/catenin system was bolstered by another recent study in which certain prostanoid receptors previously proposed to couple to $G_{12}$ proteins (15) were found to mediate the up-regulation of β-catenin-mediated transcriptional activation (16).

During the past several years, the $G_{12}$ subfamily has been implicated as a signaling component in a wide variety of cellular events. These include Rho-mediated cytoskeletal rearrangements (17–20), $Na^+/H^+$ antiporter activity (21–23), activation of c-Jun $NH_2$-terminal kinase (24–26), activation of phospholipase D (27, 28), activation of radixin (29), and signaling through certain tyrosine kinases (30–32). The disruption of $G\alpha_{13}$ by genetic knock-out has been demonstrated to result in embryonic lethality at day 9.5 (33), and a double knock-out for $G\alpha_{13}$ and $G\alpha_{12}$ is lethal at a slightly earlier point in embryogenesis (34). In addition, $G_{12}$ subfamily proteins have been revealed as potent cellular transforming agents in several studies. Human $G\alpha_{12}$ was first cloned in a screen for transforming oncogenes from a sarcoma-derived cDNA library (35), and others have shown mutationally activated variants of $G\alpha_{12}$ and $G\alpha_{13}$ to cause a transformed growth phenotype in a variety of cell culture systems (36–39). Although a direct molecular mechanism for the transforming capability of $G_{12}$ proteins has not been described, the ability of $G_{12}$ proteins noted above to induce the release of β-catenin from cadherin may be a key component of such a mechanism (13, 14).

Whereas previous studies provided evidence for the effect of $G_{12}$-cadherin interaction on "inward" signaling associated with

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cadherin function, *i.e.* re-localization of membrane-associated β-catenin to the cytoplasm and nucleus, the role of this interaction in "outward" signaling, *i.e.* cadherin-mediated cell-cell adhesion, had not yet been investigated. In this report, we demonstrate that E-cadherin-mediated cell-cell adhesion is down-regulated by mutationally activated $G_{12}$ subfamily proteins. This effect is specific to the $G_{12}$ subfamily, because other activated Gα proteins do not mimic this effect. In addition, we show that activated $G_{12}$ proteins hinder the ability of E-cadherin to prevent cell detachment and migration in a wound-filling assay. These results suggest that $G_{12}$-cadherin interaction may play an important role in cellular events leading to cancer metastasis and in cadherin-mediated multicellular events during organismal development.

## EXPERIMENTAL PROCEDURES

*Materials*—K562 human myelogenous leukemia cells were obtained from the American Type Culture Collection. MDA-MB-435 cells were obtained from Michael Kinch (Medimmune, Gaithersburg, MD). This isolate expresses neither E-cadherin nor N-cadherin. The expression vector pXEH2 encoding human E-cadherin was provided by Yutaka Shimoyama (National Okura Hospital, Tokyo, Japan). The expression vector pBATEM2 (40) encoding full-length mouse E-cadherin was obtained from Masatoshi Takeichi (Kyoto University, Kyoto, Japan). Adenovirus shuttle vector pAdTrack-CMV and adenovirus backbone vector pAdEasy-1 were provided by Bert Vogelstein (Johns Hopkins University, Baltimore, MD). The antibody for $G\alpha_{12}$ was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The antibodies for E-cadherin were purchased from Zymed Laboratories Inc. (South San Francisco, CA) and Transduction Laboratories (San Diego, CA).

*Plasmid Construction*—To engineer the ΔG12 mutant form of E-cadherin, a 650-bp *Cla*I-Bsu36I DNA fragment encoding the mouse E-cadherin cytoplasmic domain with a deletion of the 11-amino acid $G_{12}$ binding site (DQDQDYDYLNE) was produced using a two-step PCR approach. This fragment was then cloned into the E-cadherin expression vector pBATEM2 replacing the wild-type fragment and the altered region of the plasmid sequenced for verification.

*Cell Culture and Transfections*—K562 cells were maintained in RPMI 1640 medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS)[1] (Hyclone, Logan, UT) and 10 mM Hepes, pH 7.5. MDA-MB-435 cell lines were maintained in Dulbecco's modified Eagle's medium supplemented with 10% FBS and 50 μg/ml gentamicin (Invitrogen). The transfections of K562 cells were performed as follows. Approximately $10 \times 10^6$ cells were centrifuged and then resuspended in 250 μl of K562 medium. Ethanol-precipitated DNA (25 μg) was resuspended in RPMI 1640 medium without FBS and mixed with cells. The mixture was pulsed at 340 V for 10 ms using a T820 square-wave electroporator (BTX, San Diego, CA). Cells were plated in complete K562 medium and 48 h later were given 1 mg/ml Geneticin (Invitrogen). Cells were either grown in a single dish (crude transfection) or were dispensed into 96-well plates at varying dilutions (clonal transfection). For the latter condition, plates were incubated for 12 days with Geneticin replenished every 4 days. Plates with visible cell growth in <25% wells were expanded to larger wells and screened by immunoblot analysis for expression of the desired protein. MDA-MB-435 cells were transfected using FuGENE 6 (Roche Molecular Biochemicals) following the manufacturer's instructions. Cadherin constructs were co-transfected with pCMV-puro, and selection was performed using 1 μg/ml puromycin. Puromycin-resistant clones were isolated by ring cloning and screened for E-cadherin expression by immunofluorescent labeling with an anti-E-cadherin antibody.

*Recombinant Adenovirus Construction and Production*—Recombinant adenoviruses encoding mutationally activated ($^{QL}$) variants of $G\alpha_{12}$ and $G\alpha i_2$ were constructed as follows. $G\alpha_{12}{}^{QL}$ was excised from pcDNA1 using *Bam*HI and *Xba*I and directionally subcloned into the shuttle vector pAdTrack-CMV at its *Bgl*II and *Xba*I restriction sites. $G\alpha i_2{}^{QL}$ in pCMV was digested with *Eco*RI, blunted with Klenow, and re-digested with *Xba*I. The $G\alpha i_2{}^{QL}$ insert was subcloned into pAdTrack-CMV that was previously digested with *Sal*I, blunted with Klenow, and re-digested with *Xba*I. Successful recombinants of pAdTrack-CMV were verified by sequencing and then transformed by electroporation along with the adenoviral backbone vector pAdEasy-1 into BJ5183 cells. The colonies were screened for recombinant pAdEasy-1 clones, and DNA was purified using a cesium chloride density gradient. Viral production was performed as described previously (41). HEK293 cells were transfected with recombinant pAdEasy-1 DNA and grown until roughly half of the cells were detached. Cells were then subjected to 4 cycles of freeze thawing using a dry ice/methanol mixture, extracts were centrifuged at $2000 \times g$ for 5 min, and supernatants were collected and frozen. Because pAdEasy-1 recombinants contained a cDNA for green fluorescent protein (GFP) driven by a separate CMV promoter, titers of recombinant adenoviruses were estimated by infection of HEK293 cells and subsequent microscopy to visually assess green fluorescence intensity.

*Adenovirus Infections*—Cells were grown to 90% confluency in 10-cm plates, removed by the addition of trypsin, washed with phosphate-buffered saline, centrifuged at $800 \times g$ for 5 min, and then resuspended in 200 μl of phosphate-buffered saline/plate of cells. Adenoviral supernatant (20–50 μl, depending on titer) was added, and samples were incubated for 1 h at 37 °C with agitation every 10 min. Cells were resuspended in Dulbecco's modified Eagle's medium + 10% FBS and plated in T-75 flasks for aggregation assays or six-well plates for wound-filling assays.

*Cell Fast Aggregation Assay*—This assay was performed as described previously (42, 43). Cells grown as monolayers to 60–90% confluency were washed twice in HCMF buffer (10 mM Hepes, 137 mM NaCl, 5.4 mM KCl, 0.34 mM $Na_2HPO_4 \cdot 7\ H_2O$, 5.6 mM glucose, pH 7.4) supplemented with 10 mM $CaCl_2$ and then incubated in HCMF supplemented with 10 mM $CaCl_2$, 1 mM $MgCl_2$, 0.04% twice-crystallized trypsin (Worthington, Lakewood, NJ) at 37 °C for 20 min. Following FBS addition to 20% to quench trypsin, cells were pelleted at $800 \times g$ for 5 min.

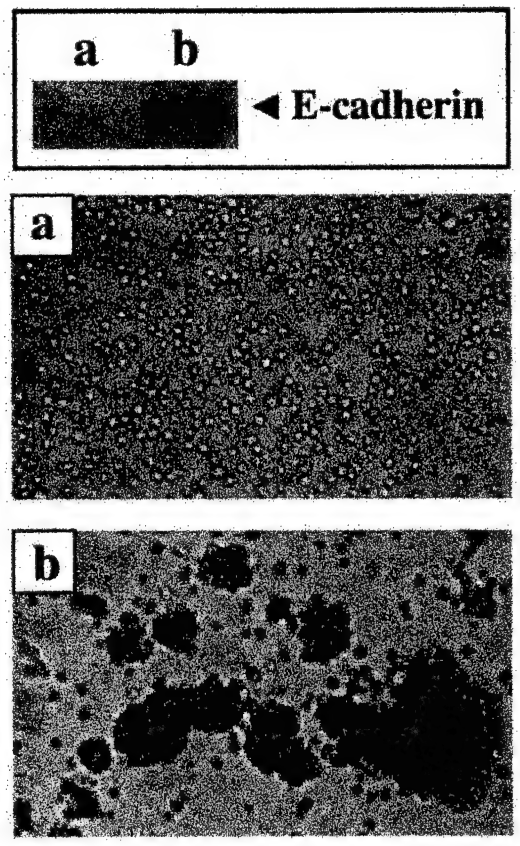


FIG. 1. **E-cadherin expression promotes cell-cell adhesion in K562 cells.** Three weeks after stable transfection with a cDNA for human E-cadherin, clonal lines of K562 cells were lysed and subjected to immunoblot analysis for E-cadherin expression. Results from two representative cell lines *a* and *b* are shown (*top panel*). Cells were examined by phase-contrast microscopy (×100 magnification) and photographed. Clonal line *a* lacking E-cadherin expression (*center panel*) and clonal line *b* exhibiting E-cadherin expression (*lower panel*) are shown.

[1] The abbreviations used are: FBS, fetal bovine serum; QL, mutationally activated variant of Gα protein; CMV, cytomegalovirus; GFP, green fluorescent protein; wt, wild type; 435 cells, MDA-MB-435 cells; 435-puro cells, 435 cells expressing control vector and selected with puromycin; 435-E-cad cells, 435 cells expressing epithelial cadherin; 435-ΔG12 cells, mutant form of E-cadherin lacking the 11-residue $G_{12}$-binding domain.

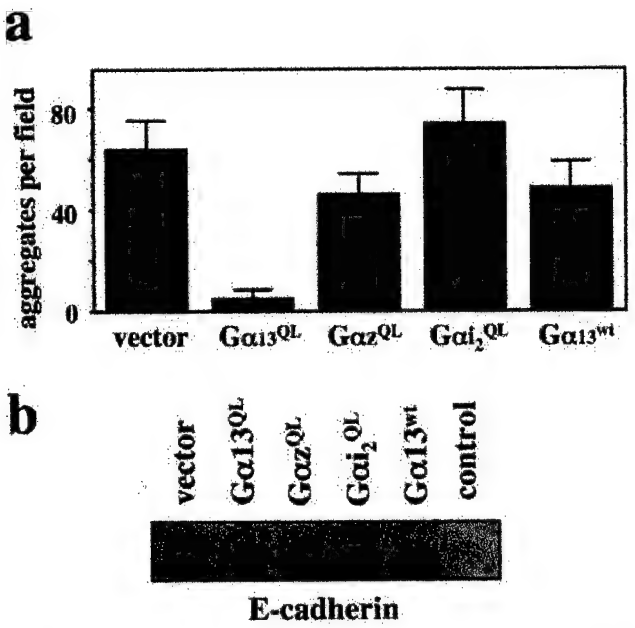


FIG. 2. **Expression of $G_{12}$ subfamily proteins prevents E-cadherin-mediated cell-cell adhesion in K562 cells.** *a*, aggregation of K562 cells expressing activated Gα subunits. K562 cells were stably transfected with the indicated Gα proteins or the plasmid pcDNA3.1 (*vector*). Clonal populations were isolated that expressed the desired Gα protein and then transfected with human E-cadherin and grown under selective pressure for 2 weeks. Cells were counted by hemacytometer to confirm that densities varied by <20% and were viewed by phase-contrast microscopy. The number of aggregates (defined under "Results") determined in five randomly selected microscopic viewfields is shown for each transfection condition. Data represent the means ± S.E. for each experimental condition and are from a single experiment that is representative of three experiments. *b*, analysis of E-cadherin expression in K562 lines chosen for study. Cells were lysed and subjected to immunoblot analysis for E-cadherin expression. The lane labeled *control* contains lysate from parental K562 cells.

Cells were resuspended in HCMF plus 10 mM $CaCl_2$ and 1 mM $MgCl_2$, DNase I was added to 0.67 mg/ml, and cells were counted using a Coulter Z1 particle counter. $2 \times 10^6$ cells next were placed in silicone-coated glass vials containing 2 ml of HCMF plus either 1 mM $CaCl_2$ or 1 mM EGTA. Vials were rotated at 90 rpm at 37 °C for 1 h, and then cell aggregation was assessed using an Eclipse® TE300 phase-contrast microscope (Nikon) at ×100 magnification. Cells were photographed using a SPOT® digital camera (Diagnostic Instruments, Sterling Heights, MI) with SPOT RT version 3.02 imaging software. For determination of the approximate size of cell aggregates, images were imported into Photoshop 4.0 (Adobe Systems, Inc.) and overlaid with a grid pattern to allow the measurement of the "surface" area of aggregates. All images were analyzed at the same scale in Photoshop, and measurements were recorded as "relative area units" for each aggregate.

*Wound-filling Assay*—Approximately 48 h post-infection, the medium was aspirated from cells, and a 20-μl pipette tip was used to scratch a pattern of vertical and horizontal "wounds" in the monolayer. Cells were washed several times with fresh medium to remove detached cells and debris, and then Dulbecco's modified Eagle's medium + 10% FBS was added to cells. Roscovitine (20 μM) (Calbiochem) was also added to reduce cell proliferation. Several wounded areas were marked for orientation and then photographed by phase-contrast microscopy as in the cell aggregation assay. At a set time ranging from 33 to 39 h in each individual experiment, marked wounds were again photographed.

## RESULTS

*$G_{12}$ Proteins Disrupt E-cadherin-mediated Cell-Cell Adhesion*—The strength of cadherin-mediated cell-cell adhesion is markedly increased by the interaction of the C-terminal cytoplasmic domain of cadherin with β-catenin (44, 45). Given the previous findings that $G_{12}$ subfamily proteins disrupt the association of cadherin and β-catenin (13, 14), we predicted that the expression of mutationally activated $G\alpha_{12}$ or $G\alpha_{13}$ might disrupt cadherin-mediated cell-cell adhesion. To test this hypothesis, we took advantage of the finding that K562 human myelogenous leukemia cells, which grow in suspension culture as free-floating cells, can be induced to form aggregates by ectopic expression of cadherin (46). K562 cells were stably transfected with a cDNA encoding human E-cadherin, and hygromycin-resistant clonal populations were selected. Consistent with the previous report (46), clonal populations that expressed E-cadherin also exhibited a dramatic change from dispersed cells to large aggregates, whereas clonal populations failing to express E-cadherin showed no such aggregation (Fig. 1).

To determine the effects of $G_{12}$ proteins on cadherin-mediated cell adhesion, we next attempted to co-express various mutationally activated ($^{QL}$) or wild-type ($^{wt}$) G protein α-subunits along with E-cadherin in K562 cells. Because of difficulties encountered in transfecting the highly aggregated E-cadherin-expressing cells, clonal K562 cell lines stably expressing various Gα proteins were first established, and then secondary transfections were performed to introduce E-cadherin. Whereas K562 cells expressing $G\alpha z^{QL}$, $G\alpha i_2{}^{QL}$, $G\alpha_{13}{}^{wt}$, or no G protein still exhibited significant aggregation, cells expressing $G\alpha_{13}{}^{QL}$ did not form aggregates (Fig. 2). These cells were essentially of the same appearance as K562 cells lacking E-cadherin (see Fig. 1). The quantitation of aggregation in the K562 cells expressing E-cadherin along with various Gα proteins was performed by visual analysis of the cell populations. For each sample, five random microscopic viewfields were selected, and the number of aggregates (defined as discrete clumps of cells that measured at ≥3-cell-diameters in breadth at the narrowest point and which remained intact as the plate was gently agitated) was counted. Cells co-transfected with E-cadherin and $G\alpha_{13}{}^{QL}$ had <10% the average number of aggregates per viewfield compared with cells co-transfected with E-cadherin and empty vector, $G\alpha_{13}{}^{wt}$, $G\alpha z^{QL}$, or $G\alpha i_2{}^{QL}$ (Fig. 2*a*). Immunoblot analysis confirmed that all transfectants expressed E-cadherin at similar levels (Fig. 2*b*). Furthermore, all of the cell aggregates formed in these experiments could be disrupted by the addition of EGTA to deplete $Ca^{2+}$ from the culture medium, providing additional evidence that the formation of aggregates was mediated by the expressed E-cadherin (data not shown).

Whereas the above data assessed cadherin-mediated cell-cell adhesion as a long term steady-state condition, we also felt it was important to examine the effect of $G_{12}$ proteins on aggregation as a short term dynamic process (42, 43, 47, 48). The chosen approach utilized an adherent cell line denoted MDA-MB-435 (435 cells), which lacks endogenous E-cadherin (see "Experimental Procedures"). These cells were stably transfected with cDNAs for either mouse E-cadherin to produce 435-E-cad cells or an empty plasmid to produce 435-puro cells, and clonal cell lines were established. A recombinant adenovirus expressing $G\alpha_{12}{}^{QL}$ along with a GFP marker was employed to introduce the activated Gα protein into the two-cell populations, whereas adenovirus expressing only GFP was used as a control. To assess short term aggregation, single cell suspensions were prepared by trypsinizing the cells in the presence of 10 mM $CaCl_2$, which preserves functional E-cadherin at the cell surface, and the cells were allowed to re-aggregate for 1 h in the presence of 1 mM $Ca^{2+}$. Under these conditions, 435-E-cad cells infected with control adenovirus rapidly formed large aggregates (Fig. 3*a*). However, the expression of $G\alpha_{12}{}^{QL}$ in the 435-E-cad cells markedly hindered the formation of the large cell aggregates. Instead, these cells formed distinctly smaller aggregates that were much greater in number (Fig. 3*a*). Furthermore, 435-puro cells infected with either control adenovirus or control containing $G\alpha_{12}{}^{QL}$ did not form aggregates.

To directly assess the importance of interaction between E-cadherin and $G_{12}$ proteins in the observed $G\alpha_{12}$-mediated

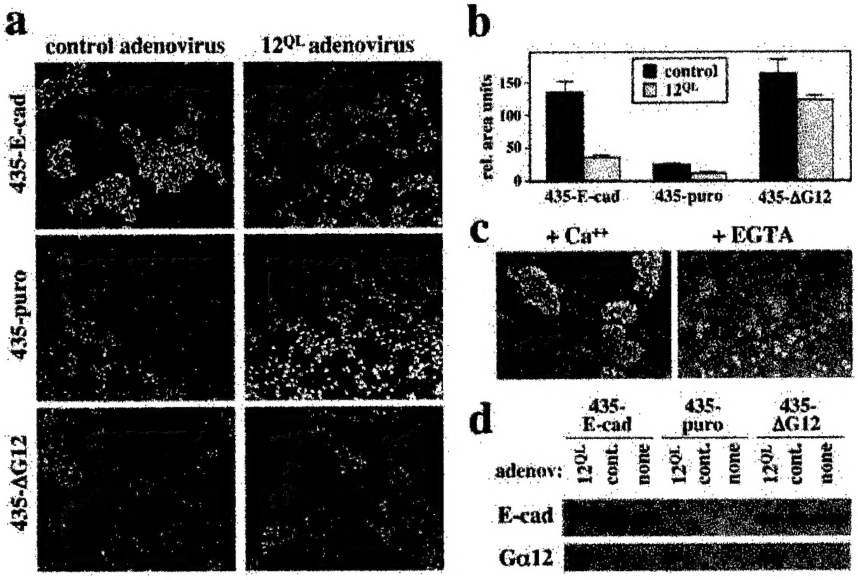


FIG. 3. **$G_{12}$ subfamily proteins acutely hinder E-cadherin-mediated cell aggregation.** *a*, effect of introduction of activated $G\alpha_{12}$ on aggregation of engineered 435 cells. Cells stably expressing E-cadherin (*435-E-cad*), a control plasmid (*435-puro*), or E-cadherin lacking the $G_{12}$-interacting domain (*435-ΔG12*) were infected with recombinant adenovirus expressing mutationally activated $G\alpha_{12}$ ($12^{QL}$) or with a control recombinant adenovirus. Cells were normalized to identical densities and then subjected to fast aggregation assays (see "Experimental Procedures"). Cells photographed 1 h after initiation of aggregation are shown. *b*, quantitation of aggregate size. Following fast aggregation assays, two distinct viewfields for each experimental condition in *a* were photographed, and the three largest aggregates per viewfield were measured (see "Experimental Procedures"). Data were recorded as relative (*rel.*) area units and are presented as the means ± S.E. *c*, calcium requirement for aggregation of 435-E-cad cells. Cells that received either 1 mM $Ca^{2+}$ or 1 mM EGTA during the 1-h aggregation period are shown. *d*, expression of E-cadherin and $G\alpha_{12}$ in the engineered 435 cells chosen for study. Cell lines infected with the indicated recombinant adenoviruses ($12^{QL}$ or *cont.*) or no adenovirus (*none*) were lysed and subjected to immunoblot analysis for E-cadherin expression and $G\alpha_{12}$ expression.

inhibition of cadherin-dependent aggregation, we took advantage of a previous study (14) that mapped the $G_{12}$-interacting region of human E-cadherin to a highly charged 11-residue domain near the C terminus. MDA-MB-435 cells were engineered to stably express a mouse E-cadherin cDNA lacking this 11-residue region (denoted 435-ΔG12 cells), and a clonal line of these cells with E-cadherin levels similar to the levels in 435-E-cad cells (Fig. 3*d*) was chosen for the study. When subjected to the fast aggregation assay described above, 435-ΔG12 cells formed large aggregates (Fig. 3*a*) that were indistinguishable from 435-E-cad cells (*i.e.* those expressing full-length E-cadherin). However, the expression of $G\alpha_{12}{}^{QL}$ in the 435-ΔG12 cells did not change the appearance or size of these aggregates (Fig. 3*a*), which contrasted sharply with the results observed in 435-E-cad cells. The quantitation of the approximate size of these cell aggregates also revealed a sharp $G\alpha_{12}{}^{QL}$-induced decrease in aggregate size in 435-E-cad cells but not a significant decrease in 435-ΔG12 cells (Fig. 3*b*). These findings suggest that the binding of activated $G\alpha_{12}$ directly to cadherin is required for the disruption of cell-cell adhesion. The addition of EGTA instead of $Ca^{2+}$ during the aggregation assay caused complete dissociation of 435-E-cad cells (Fig. 3*c*) and 435-ΔG12 cells (data not shown), providing additional evidence that cell aggregation was mediated through E-cadherin. Furthermore, an immunoblot analysis confirmed that $G\alpha_{12}$ was expressed at similar levels in all cells infected with the $G\alpha_{12}{}^{QL}$ adenovirus and also showed that neither $G\alpha_{12}{}^{QL}$ expression nor adenovirus infection significantly altered E-cadherin levels (Fig. 3*d*). Taken together with the data presented in Fig. 2, these findings provide compelling evidence that the $G_{12}$ subfamily indeed down-regulates E-cadherin-mediated cell-cell adhesion.

*$G_{12}$ Proteins Counteract Cadherin-mediated Inhibition of Cell Migration*—An important property of cadherins is the maintenance of tissue integrity; this property is pertinent not only to normal solid tissues but also to neoplastic tissues (2, 49). Wound-filling assays have been commonly used to assess

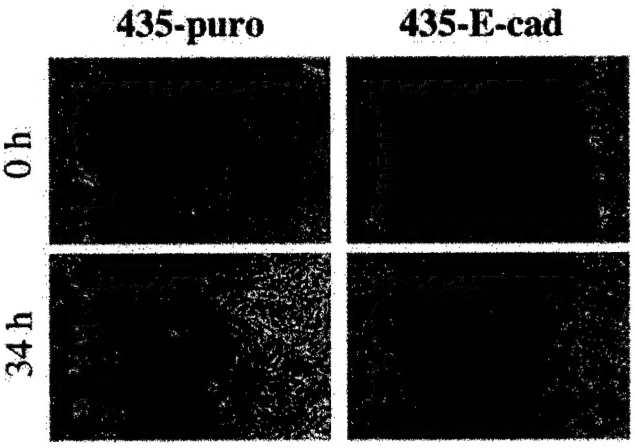


FIG. 4. **Expression of E-cadherin prevents migration of individual 435 cells.** Wound-filling assays (see "Experimental Procedures") using 435-puro and 435-E-cad cells are shown. *Upper panels* were photographed at time of initial wounding, wounds were marked for orientation, and *lower panels* were photographed 34 h after wounding.

the ability of cadherins to modulate cell migratory and invasive phenotypes (50, 51). To determine whether $G_{12}$ proteins could influence this property of cadherins as well, we utilized 435-E-cad cells and 435-puro cells in wound-filling assays (see "Experimental Procedures"). When a monolayer of cells was wounded by scraping, cells lacking E-cadherin (435-puro cells) frequently broke free from the advancing "wall" of cells and migrated into the wound area as lone cells (Fig. 4). For the purposes of this assay, we designated such cells as "free-migrating cells." Conversely, cells expressing E-cadherin (435-E-cad cells) advanced slowly into the wound area as a cohesive wall of cells in which cells at the leading edge maintained contact with other cells (Fig. 4). To test the effect of $G_{12}$ proteins on this process, we again utilized recombinant adenovi-

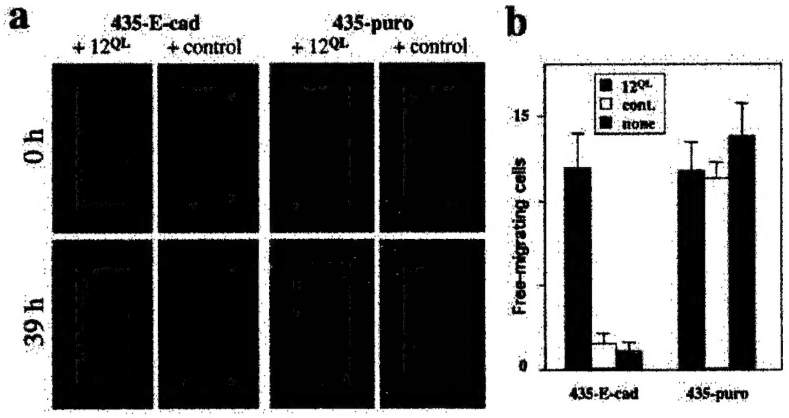


FIG. 5. **$G_{12}$ subfamily proteins overcome E-cadherin-mediated suppression of cell migration.** *a*, effect of activated $G\alpha_{12}$ on E-cadherin-mediated inhibition of cell migration. Wound-filling assays (see "Experimental Procedures") were performed using the indicated 435 cells infected 40 h earlier with recombinant adenoviruses expressing the mutationally activated variant of either $G\alpha_{12}$ ($12^{QL}$) or $G\alpha i_2$ (*control*). Cells visualized by fluorescence microscopy for GFP expression (indicative of successful adenovirus infection) are shown. *Upper panels* were photographed at time of initial wounding, and *lower panels* were photographed 39 h after wounding. *b*, quantitation of the effect of activated $G\alpha_{12}$ on wound-filling behavior of 435 cells. For the indicated 435 cells infected with activated $G\alpha_{12}$ adenovirus ($12^{QL}$), control adenovirus (*cont.*), or no adenovirus (*none*), the number of free-migrating cells (defined under "Results") was determined 39 h after wounding. Data from five randomly selected viewfields are presented as the means ± S.E. for each condition. A single experiment that is representative of four experiments is shown.

ruses to introduce either $G\alpha_{12}{}^{QL}$ or, as a control, $G\alpha i_2{}^{QL}$ into these cells and repeated the wound-filling assays. Whereas infection with the control adenovirus did not alter the wound-filling behavior of 435-E-cad cells, the infection with adenovirus expressing $G\alpha_{12}{}^{QL}$ caused 435-E-cad cells to behave as free-migrating cells (Fig. 5*a*). The free-migrating behavior of 435-puro cells, which lack E-cadherin, was unchanged by infection with either of the adenoviruses producing $G\alpha$ proteins (Fig. 5*a*). The recombinant adenoviruses used in these studies contain a cDNA for GFP governed by a separate promoter from that which drives $G\alpha$ protein expression. This feature allows the investigator to gauge the efficiency of adenoviral infection by monitoring live cells for green fluorescence (41). When the entire cell population was observed by fluorescence and phase-contrast microscopy, it was clear that essentially all cells were expressing GFP (data not shown), indicating that the efficiency of adenoviral infection approached 100% for the experimental conditions employed.

The results of the wound-filling assays were quantitated through a visual analysis. Roughly 36 h after wounding, five distinct viewfields per sample were randomly selected, and the number of free-migrating cells was determined. To differentiate between live substrate-adherent cells and dead cells/debris, plates were gently agitated, and only cells that remained firmly attached to the dish were counted. As shown in Fig. 5*b*, free-migrating 435-E-cad cells seldom were observed; however, the introduction of $G\alpha_{12}{}^{QL}$ into 435-E-cad cells increased the number of free-migrating cells to a level similar to that observed in 435-puro cells. Neither the infection of 435-E-cad cells with control adenovirus nor the expression of $G\alpha_{12}{}^{QL}$ in 435-puro cells caused any significant increase in the number of free-migrating cells (Fig. 5*b*), indicating that the effects of $G\alpha_{12}$ in this experimental system are indeed the result of disruption of E-cadherin function.

*$G_{12}$-mediated Effect on Cadherin Function Is Not Dependent on Rho Activation*—There is considerable evidence that $G_{12}$ proteins signal at least in part through the activation of the Rho family of monomeric G proteins (17, 19). The Rho pathway would not be expected to contribute to the disruption of cell aggregation reported here, because Rho is well documented as a positive regulator of cadherin-mediated cell-cell adhesion (52–54). However, because Rho-mediated signaling has been implicated as playing a role in cell migration and metastasis (55, 56), we considered it important to confirm that the consequences of $G_{12}$ signaling on cadherin-mediated suppression of migration were not the result of the "Rho arm" of the $G_{12}$ pathway. Therefore, we conducted wound-filling assays in the presence of the Rho kinase inhibitor Y27632. As shown in Fig. 6*a*, the expression of $G\alpha_{12}{}^{QL}$ caused 435-E-cad cells to behave as free-migrating cells, and this was not altered in the presence of 10 $\mu$M Y27632. In 435-E-cad cells infected with control adenovirus, essentially no free-migrating cells were observed in the presence or absence of Y27632. As described above, we randomly selected five viewfields for each experimental condition and counted free-migrating cells. In 435-E-cad cells infected with $G\alpha_{12}{}^{QL}$ adenovirus or control adenovirus, Y27632 did not cause a significant change in the average number of free-migrating cells (Fig. 6*b*). Y27632 also had no effect on the behavior of 435-puro cells (Fig. 6*b*, data not shown in Fig. 6*a*). The Y27632 used in this study was effective in blocking a known $G_{12}$-to-Rho signaling process in 435 cells, that being the ability of $G\alpha_{12}{}^{QL}$ to induce cell rounding (20, 57, 58). In 435-E-cad cells, $G\alpha_{12}{}^{QL}$ induced nearly all of the cells (80.3 ± 8.8%) to assume a distinctly rounded morphology. However, in the presence of Y27632, $G\alpha_{12}{}^{QL}$ failed to induce cell rounding at a percentage significantly higher than background (Fig. 6*c*). These results confirm that the effect of activated $G\alpha_{12}$ on cadherin-mediated inhibition of cell migration is not mediated through an effect on Rho signaling.

## DISCUSSION

The discovery that $G_{12}$ proteins bind to the cytoplasmic region of cadherins and cause the release of $\beta$-catenin revealed an unexpected link between heterotrimeric G proteins and $\beta$-catenin-mediated transcriptional activation (13). The subsequent mapping of the $G\alpha_{12}$ binding and $\beta$-catenin binding regions on cadherin confirmed a requirement for direct $G_{12}$-cadherin interaction in this process and allowed the dissection of the two interactions and their respective roles in regulating the downstream signaling capacity of $\beta$-catenin (14). Because the cell-cell adhesive function of cadherins is known to be dependent on $\beta$-catenin interaction with the cadherin cytoplasmic domain (44), it seemed reasonable to suspect that the binding of $G_{12}$ proteins to cadherin might exert down-regulatory control over cadherin-mediated cell-cell adhesion. In this study, we tested this hypothesis by utilizing several model systems that allow the measurement of extracellular function of cadherin in its capacity to promote cell aggregation and to hinder detachment

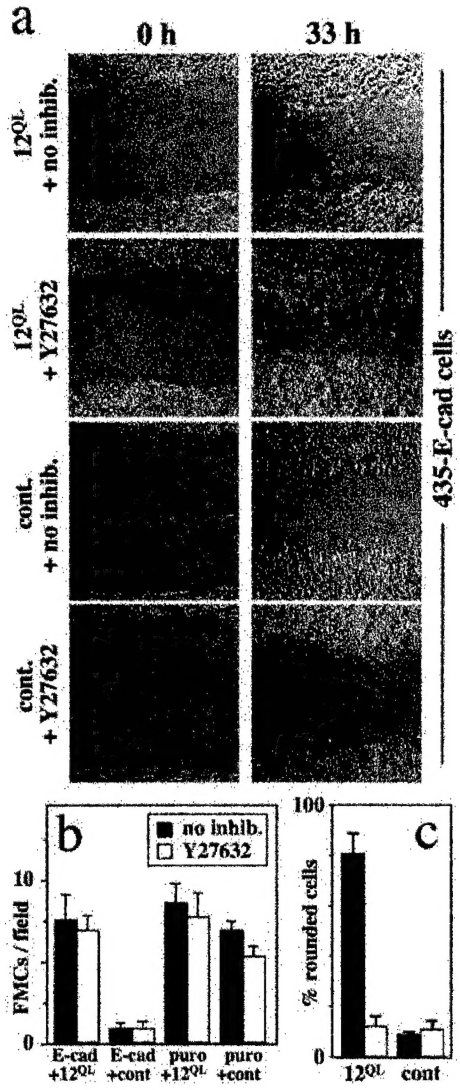


FIG. 6. **The effect of $G_{12}$ on E-cadherin function is not Rho-mediated.** *a*, effect of activated $G\alpha_{12}$ on wound-filling behavior is not affected by Y27632. Results of wound-filling assays are shown in which 435-E-cad cells previously infected with mutationally activated $G\alpha_{12}$ adenovirus ($12^{QL}$) or control adenovirus (*cont.*) were wounded, marked for orientation, immediately photographed (*left panels*), and given culture medium containing 10 $\mu$M Y27632 (*Y27632*) or no additive (*no inhib.*). Wounds were re-photographed after 33 h (*right panels*). *b*, quantitation of wound-filling results. The number of free-migrating cells (labeled *FMCs* and defined under "Results") was determined in five randomly selected microscopic viewfields for each adenovirus infection condition ±Y27632 treatment in 435-E-cad (*E-cad*) and 435-puro (*puro*) cells. Data represent means ± S.E. for each condition and are from a single experiment that is representative of three experiments. *c*, effect of Y27632 on $G\alpha_{12}$-induced rounding of 435-E-cad cells. Cells grown on coverslips and infected 40 h earlier with mutationally activated $G\alpha_{12}$ adenovirus ($12^{QL}$) or control adenovirus (*cont*) received culture medium containing 10 $\mu$M Y27632 (*open bar*) or no inhibitor (*closed bar*), and after 28 h, cells were washed with phosphate-buffered saline, fixed in 4% paraformaldehyde, and observed by phase-contrast microscopy. The percentage of rounded cells among total cells was determined for three randomly selected viewfields, and the means ± S.E. are shown for each condition.

and migration of individual cells from the surrounding cells. Our results clearly demonstrate that the $G_{12}$ subfamily proteins $G\alpha_{12}$ and $G\alpha_{13}$ are able to markedly influence these functions of cell-surface cadherins.

The activation of Rho is the most well defined of the known $G_{12}$-mediated signaling pathways, because $G_{12}$ proteins have been shown to directly stimulate certain guanine nucleotide exchange factors that can then trigger Rho activation (59–61). Because Rho signaling through Rho kinase has been demonstrated to promote cell migration in several experimental systems (55, 56), we used the compound Y27632 to specifically inhibit Rho kinase and then examined whether activated $G\alpha_{12}$ could still down-regulate cadherin function in wound-filling assays. The results clearly showed that Y27632 did not hinder the ability of activated $G\alpha_{12}$ to counteract cadherin function, but Y27632 still potently disrupted cell rounding induced by activated $G\alpha_{12}$, which is a signaling event mediated through Rho and Rho kinase (20, 57, 58). These results confirmed that the effects of $G_{12}$ proteins on cadherin function occur in a manner independent of Rho activation.

The role of cadherin as a tumor suppressor is well documented (10, 45, 49). In addition, several reports have demonstrated the role of $G\alpha_{12}$ and $G\alpha_{13}$ in cellular transformation (35, 36, 39). Therefore, it is an attractive model that the transforming capacity of activated $G_{12}$ proteins is at least in part the result of their ability to associate with cadherins. We can envision at least two ways in which this interaction, which clearly leads to destabilization of $\beta$-catenin binding (13, 14), could promote an oncogenic phenotype. First, the release of $\beta$-catenin from a membrane-associated state and its subsequent translocation to the nucleus would allow the binding of $\beta$-catenin to the transcription factor TCF and activation of key growth-regulating genes (62). Second, the disruption of cadherin extracellular adhesive function by $G_{12}$ proteins could accelerate the transition of a tumor to a metastatic invasive state. Our results in cell aggregation assays and wound-filling assays provide support for such a model in which an activating mutation within a $G_{12}$ protein could result in constitutive down-regulation of cadherin function.

Taken together with our previously reported results, the current finding that $G_{12}$ proteins down-regulate cadherin function provides new insight into not only $G_{12}$-mediated cellular transformation but possibly a broad array of cellular events that require cadherin-mediated adhesion (or down-regulation thereof) among cells in a population. The modulation of cadherin function is critical to such early developmental events as axonal patterning, cell migration, cell sorting, epithelial-to-mesenchymal transition, and maintenance of epithelial integrity (4). The disruption of $G_{12}$-cadherin interaction in organismal model systems will undoubtedly be valuable in defining the physiological significance of the interaction. *Drosophila* may be one particularly useful system, because flies lacking the $G\alpha_{12}$/$G\alpha_{13}$ ortholog Concertina are impaired in developmental processes as early as gastrulation (63), and cadherins are also known to play a role in several stages of early embryogenesis (4).

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